# Amino Acid and Monosaccharide Transporters of Human Small Intestine

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# 1. Summary

Intestinal trans-cellular amino acid and monosaccharide absorption is a two-step process mediated by different transport proteins located in the apical (facing the lumen of the gut) and the basolateral (facing the extracellular space) membrane of small intestinal enterocytes[1-3].

## 1.1. Human intestine luminal ACE2 and amino acid transporters are regulated by ACE-inhibitors

$B^0AT1$ (SLC6A19) is a sodium-dependent neutral amino acid transporter expressed in the luminal membrane of small intestinal enterocytes and of proximal tubule kidney cells[1] whose deficiency causes Hartnup disorder[4]. Small intestinal $B^0AT1$ expression depends on the presence of the carboxymonopeptidase ACE2 (angiotensin converting enzyme 2)[5], an enzyme that belongs to the renin-angiotensin system and whose expression is induced in many tissues by application of RAS-active medications including ACE-inhibitors (ACEIs) and angiotensin II $AT_1$ receptor blockers (ARBs)[6-8]. A similar interaction of the IMINO transporter SIT1 (sodium-dependent imino transporter 1; SLC6A20) – a small intestinal and proximal tubule L-proline (Pro) transporter – and ACE2 has been proposed[9], but not demonstrated yet.

We have shown, using the *Xenopus laevis* oocytes expression system, that human ACE2 functionally interacts with SIT1 and that both amino acid transporters ($B^0AT1$ and SIT1) co-localize with ACE2 to the brush border membrane (BBM) of small intestinal enterocytes. In addition, we showed that treatment with ACEIs increases small intestinal expression of ACE2, of amino acid transporters $B^0AT1$ and PAT1 (SLC36A1) and of peptide transporter PEPT1 (SLC15A1). We further demonstrated the axial distribution of several amino acid transporters/transport units expressed in the luminal and basolateral enterocyte membrane in one single cohort of human patients.

## 1.2. Intestinal monosaccharide transporter expression in diabetic patients – effect of metformin treatment

Noninsulin-dependent diabetes mellitus (NIDDM) is a common disease of glucose metabolism that may result in severe morbidity[10]. The dimethylbiguanide metformin is the first-line antihyperglycemic medication in NIDDM treatment[11]. Metformin increases insulin-stimulated glucose (Glc) uptake in muscle cells and decreases hepatic Glc output[12,13]. Furthermore, metformin treatment has an inhibitory effect on intestinal glucose absorption as shown in rodents[14]. Three different small intestinal hexose transporters, SGLT1 (SLC5A1),

GLUT2 (SLC2A2) and GLUT5 (SLC2A5) mediate intestinal monosaccharide absorption with SGLT1 being a sodium-dependent BBM glucose (Glu) and galactose (Gal) transporter, and GLUT2 and 5 being facilitated diffusion proteins with GLUT2 transporting Glu, Gal and fructose (Frc) and GLUT5 transporting Frc only[15-17]. Whereas GLUT5 is expressed in the BBM, GLUT2 is expressed in the basolateral enterocyte membrane, but shows luminal staining in conditions of experimental diabetes, in fructose- or glucose rich diets in rodents[18], as well as in morbidly obese human subjects[19]. Whereas specific fructose- and sucrose rich diets increase small intestinal SGLT1, GLUT2 and GLUT5 expression[17], the changes in small intestinal hexose transporter expression as a result of NIDDM seems to be more complex[16,20].

We could show an increase in small intestinal GLUT2 mRNA expression in non-treated human type 2 diabetics, an effect that is abolished upon metformin treatment. Whereas SGLT1 expression was not affected by NIDDM, also GLUT5 gene expression was non-significantly increased in non-treated human type 2 diabetics. We could further show the longitudinal gene expression of hexose transporters along the human digestive tract, as well as protein localization of facilitated diffusion protein GLUT5.

## 1.3. The molecular mechanism of intestinal levodopa absorption and its possible implication on treatment of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder mainly caused by dopamine depletion in the substantia nigra, clinically manifesting by different symptoms including its hallmark, the trias of bradykinesia, resting tremor and rigidity[21]. Since its introduction in 1968, levodopa (L-Dihydroxyphenylalanine, L-Dopa) is the major therapeutic agent in treating PD[22]. After passing the Blood Brain Barrier (BBB), levodopa is converted by the dopa-decarboxylase into dopamine[23]. To prevent levodopa metabolism prior to its transport across the BBB, orally administered levodopa is routinely given in combination with a DDC inhibitor (DDCI) (as e. g. carbidopa or benserazide) that may be combined with a cathecol-o-methyl transferase (COMT) inhibitor (such as entacapone), with the latter avoiding levodopa methylation into 3-0-methyldopa[22]. Levodopa is a large neutral amino acid (LNAA) showing structural homology to the aromatic amino acids (AAs) L-phenylalanine (Phe) and L-tyrosine (Tyr). Whereas levodopa is transported via the cysteine and dibasic AA exchanger $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) across the luminal enterocyte membrane[24], three different AA transporters were shown to carry levodopa across the basolateral membrane, namely the $Na^+$-independent L-type amino acid exchangers LAT1 (SLC7A5) and LAT2 (SLC7A8)[25], as well as the aromatic AA uniporter TAT1 (SLC16A10)[26]. In addition to $b^{0,+}$AT-rBAT, other luminal AA transporters including the broad neutral amino acid transporter $B^0$AT1 (SLC6A19) have been proposed to transport levodopa[24].

Testing different candidate transporters, we could show, that levodopa transport across the

luminal enterocyte membrane is restricted to the neutral and dibasic AA exchanger $b^{0,+}$AT-rBAT. Luminal neutral AAs decreased intestinal levodopa absorption, whereas luminal DDC- (Carbidopa and Benserazide) and COMT inhibitors (Entacapone) did not affect levodopa transport. High intra- and extracellular AA concentrations (mimicking the postabsorptive state) had no effect on levodopa absorption. Basolateral TAT1 displayed a major role in levodopa efflux from intestinal enterocytes. Whereas no gender differences in intestinal levodopa transporter expression were seen, the gene- and protein expression of levodopa transporters followed a circadian rhythm.

# 2. Zusammenfassung

Die Absorption von Aminosäuren und Monosacchariden im Dünndarm durch die apikale (dem Darmlumen zugewandte Seite) und die basolaterale (dem Extrazellulärraum zugewandte Seite) Enterozytenmembran wird durch das Zusammenspiel verschiedener Transportproteine gewährleistet[1-3].

## 2.1. ACE-Hemmer steigern die Expression intestinaler Aminsäurentransporter sowie der Peptidase ACE2 im menschlichen Dünndarm

$B^0AT1$ (SLC6A19) bezeichnet einen natriumabhängigen Transporter neutraler Aminosäuren, der in der luminalen Membran intestinaler Enterozyten sowie des proximalen Tubulusepithels exprimiert ist[1]. Die $B^0AT1$ Defizienz führt zur Hartnup-Krankheit[4]. Die intestinale Expression von $B^0AT1$ in der luminalen Enterozytenmembran bedingt das Vorhandensein der RAS (Renin Angiotensin System) Peptidase ACE2 (Angiotensin Converting Enzym 2)[5], einem Enzym, dessen Expression durch die Applikation von ACE-Hemmern oder Sartanen in verschiedenen Geweben induziert wird[6-8]. In ähnlicher Weise wie $B^0AT1$ und ACE2 scheinen auch ACE2 und SIT1 (SLC6A20) – der natriumabhängige IMINO Transporter 1 – zu interagieren. Diese Interaktion konnte bisher allerdings noch nicht eindeutig aufgezeigt werden[9].
Wir konnten in einem heterologen Expressionssystem (*Xenopus laevis* Oozyten) nachweisen, dass ACE2 und SIT1 funktionell interagieren. Weiter konnten wir zeigen, dass beide genannten Aminosäurentransporter ($B^0AT1$ und SIT1) mit dem akzessorischen Protein ACE2 in der Bürstensaummembran des humanen Dünndarmepithels exprimiert sind. Interessanterweise zeigten Patienten unter Dauermedikation mit ACE-Hemmern eine vermehrte intestinale Genexpression von ACE2, $B^0AT1$, des luminalen Aminosäurentransporters PAT1 (SLC36A1) und des Peptidtransporters PEPT1 (SLC15A1). Weiter konnten wir die Genexpression mehrerer Aminosäurentransporter entlang des humanen Gastrointestinaltrakts innerhalb einer einzigen Patientenkohorte aufzeigen.

## 2.2. Expression intestinaler Monosaccharidtransporter bei Typ 2 Diabetikern und der Einfluss der Metformintherapie

Diabetes mellitus Typ 2 (= NIDDM) ist eine häufige Erkrankung des Glukosestoffwechsels, die mit schwerwiegenden klinischen Manifestationen einhergehen kann[10]. Das Dimethylbiguanid Metformin wird als Standardtherapie des NIDDM verwendet[11]. Metformin erhöht hierbei die Insulinvermittelte Glukoseaufnahme in die Muskelzellen und vermindert die hepatische Glukoseexkretion[12,13]. Des weiteren führt die Applikation von Metfmormin zu einer verminderten intestinalen Glukoseabsorption im Tiermodell[14]. Drei Transporter sind

für die intestinale Glukoseabsorption verantwortlich: SGLT1 (SLC5A1), GLUT2 (SLC2A2) und GLUT5 (SLC2A5). SGLT1 bezeichnet einen in der luminalen Enterozytenmembran exprimierten natriumabhängigen Glukose- (Glc) und Galaktose (Gal) Transporter. GLUT2 und -5 sind Diffusionsproteine. GLUT5 transportiert Fruktose (Frc) und ist – analog SGLT1 – in der luminalen Enterozytenmembran exprimiert. GLUT2 transporteriert alle drei Monosaccharide (Glc, Gal und Frc) durch die Basalmembran[15-17]. Eine GLUT2 Expression in der luminalen Enterozytenmembran konnte im Tiermodell bei Ratten mit Diabetes mellitus, sowie nach fruktose- oder glukosereicher Diät beobachtet werden[18]. In einer einzigen Humanstudie konnte ebenfalls eine apikale GLUT2 Expression bei adipösen Probanden gezeigt werden[19]. Während eine fruktose- und sukrosereiche Diät im Tiermodell zu einer vermehrten intestinalen SGLT1, GLUT2 und GLUT5 führt[17], wurde der Einfluss des Typ 2 Diabetes auf die intestinale Hexosetransporterepression noch nicht ausreichend untersucht[16,20].

Wir konnten bei Patienten mit Typ 2 Diabetes ohne Metformintherapie eine vermehrte intestinale GLUT2 Genexpression im Vergleich zu Nichtdiabetikern nachweisen. Die GLUT2 Expression bei Typ 2 Diabetikern unter Metformintherapie war interessanterweise unverändert zu den Nichtdiabetikern. Ein ähnlicher, allerdings statistisch nicht signifikanter Effekt zeigte die intestinale GLUT5 Genexpression (indem ebenfalls metformintherapierte Diabetiker im Vergleich zu Nichtdiabetikern und Diabetikern ohne Metformin eine Tendenz zu erhöhter intestinaler GLUT5 Genexpression zeigten). Im Gegensatz dazu war die SGLT1 mRNA gleichmässig bei behandelten- und nicht behandelten Diabetikern sowie bei Nichtdiabetikern exprimiert. Desweiteren konnten wir die Genexpression der Monosaccharidtransporter GLUT2, GLUT5 und SGLT1 entlang des humanen Darms innerhalb einer einzigen Patientenkohorte aufzeigen.

## 2.3. Der molekulare Mechanismus der intestinalen Levodopa Absorption und mögliche Auswirkungen auf die Behandlung des Morbus Parkinson

Morbus Parkinson ist eine neurodegenerative Erkrankung, die durch Absterben der Dopamin produzierenden Nervenzellen in der Substantia Nigra verursacht wird. Klinisch manifestiert sich die Parkinsonkrankheit typischerweise durch die Trias: Rigor, Bradykinesie und Ruhetremor[21]. Levodopa (L-Dihydroxyphenylalanine, L-Dopa) stellt seit seiner Markteinführung im Jahr 1968 das am meisten verwendete Medikament zur Behandlung der Parkinsonkrankheit dar[22]. Levodopa wirkt dopaminerg, indem es nach Transport durch die Blut-Hirn-Schranke durch die DOPA-Decarboxylase (DDC) zu Dopamin metabolisiert wird[23]. Um einen vorzeitigen (vor Transport durch die Blut-Hirn-Schranke) Levodopa Metabolismus zu verhindern, wird Levodopa routinemässig in Kombination mit eine DDC Inhibitor (z.B. Carbidopa oder Benserazid) verabreicht. Diese Kombination kann weiter um einen Catechol-O-Metyhltransferase Inhibitor (z.B. Entacapon) ergänzt werden, der eine

Levodopa Methylierung verhindern soll[22]. Strukturell ist Levodopa eine unpolare Aminosäure, die mit den Aminosäuren Leucin, Phenylalanin und Tyrosin verwandt ist und die via den Aminsäurenaustauscher $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) durch die luminale Enterozytenmembran transportiert wird[24]. Während andere neutrale Aminsäurentransporter (wie z.B. $B^0$AT1 (SLC6A19)) als mögliche (zusätzliche) Levodopatransporter der apikalen Enterozytenmembran vorgeschlagen wurden[24], sind drei verschiedene Transporter für den basolateralen Levodopaefflux aus dem Enterozyten verantwortlich, nämlich die Aminosäurenaustauscher LAT1 (SLC7A5) und LAT2 (SLC7A8)[25], sowie der aromatische Uniporter TAT1 (SLC16A10)[26].

Wir testeten verschiedene luminale Aminosäurentransporter ($b^{0,+}$AT-rBAT, SIT1, PAT1, ASCT2 und $B^0$AT1) auf einen möglichen Levodopa Transport. Hierbei zeigte sich, dass einzig der luminale Aminsäurenaustauscher $b^{0,+}$AT-rBAT Levodopa tansportiert. Die gleichzeitige verabreichung luminaler Aminosäuren reduzierte die Levodopa absorption, wohingegen die zeitgleiche Gabe von DDC Inhibitoren (Carbidopa oder Benserazid) oder des COMT Inhibitors Entacapon keinen Einfluss auf den Levodopatransport hatten. Hohe intra- oder extrazelluläre Aminosäurekonzentrationen (entsprechend der postabsorptiven Phase der Verdauung) hatten keinen Effekt auf die Levodopaaufnahme. Der basolaterale Uniporter TAT1 scheint essentiell für den Levodopaefflux aus intestinalen Enterozyten zu sein. Die Gen- und Proteinexpression der Levodopatransporter ist geschlechtsunabhängig, zeigt aber eine Korrelation zum zirkadianen Rhythmus.

# 3. Nutrient absorption in the small intestine

## 3.1. The small intestine architecture

The human small intestine is characterised by an epithelium forming a continuous layer of cells organized into villi protruding into the lumen of the gut (Figure 1a) and crypts that invaginate into the underlying tissue[27]. The total surface of the small intestine is estimated being about 200$m^2$[28]. Along the gastrointestinal tract, all segments are divided into four layers: the mucosa (epithelium, lamina propria, and muscular mucosae), the submucosa, the muscularis propria (inner circular muscle layer, and outer longitudinal muscle layer), and the serosa. The layer facing the intestinal lumen consists of epithelial cells (see below), that is attached to the basement membrane overlying the lamina propria, consisting of connective tissue and lymph nodes. The third and deepest layer of the mucosa is the muscularis mucosae, a continuous film of smooth muscle cells. The mucosa resides on top of the submucosa that contains a variety of inflammatory cells, lymphatics, autonomic nerve fibers, and ganglion cells, and overlies the muscularis propria. The muscularis propria consists of an inner circular layer and an outer longitudinal layer. In between reside autonomic neural fibers and ganglionic clusters that form a myenteric plexus. Peristaltic waves of the muscularis propria propel food along the GI tract. The serosa (consisting of an outer layer of squamous epithelial cells, referred to as mesothelium) or the adventitia (lacking an outer layer of mesothelial cells) build the outmost GI layers [29] (Figure 1b).

**Figure 1a**

**Figure 1b**

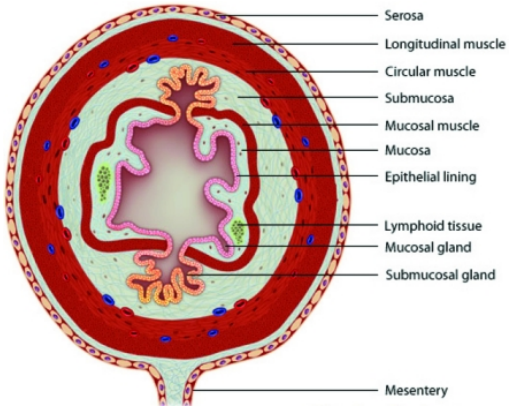


**Figure 1a. Duodenal villus.** Seen is a continuous layer of enterocytes interspersed by goblet cells (20x magnification; hematoxylin-eosin staining) (from [30]). **Figure 1b. Arrangement of small intestinal layers.** The wall of the gastrointestinal tract is organised in a mucosal-, a submucosal-, a musclular and a serosal layer (from [31]).

## 3.2. The intestinal epithelium

The intestinal epithelium is the fastest regenerating tissue within the human body, completely renewing within 5 days (with exception of Paneth cells)[32]. The intestinal crypts contain Paneth cells that synthesize antimicrobial peptides and stem cells that migrate out of the crypts onto the villi differentiating into enterocytes (providing inter alia nutrient absorption), Goblet cells (producing a protective mucus layer) and enteroendocrine cells (secreting intestinal hormones)[27,32-34]. Two opposing stem cell models are described in the literature: The '*+4 position'* model assumes, that the crypt base is solely populated by Paneth Cells and the +4 stem cells locate just above the Paneth cells at the +4 position (Figure 2, '*+4 position'* model). Another (later described) theory assumes, that crypt base columnar (CBC) cells that are intercalated between Panth cells at the crypt base build the true intestinal stem cells (Figure 2, '*stem cell zone'* model)[35].

**Figure 2**

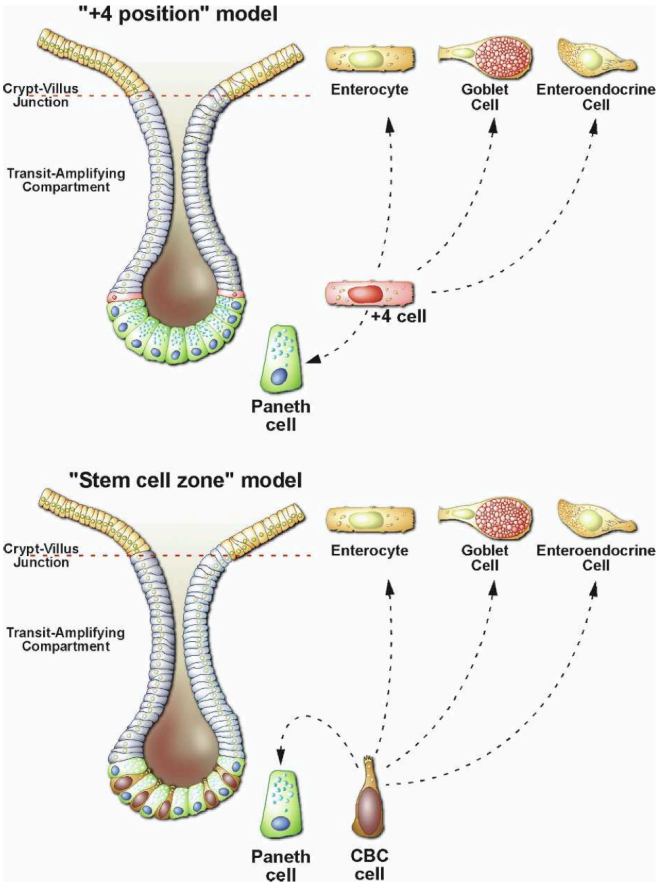


**Figure 2. Intestinal stem cell models.** Schematic representation of the crypt-to-villus axis (left) and of the differentiation of small intestinal stem cells (right). The upper panel depicts the *'+4 position'* stem cells model and the lower panel shows the *'Stem cell zone'* model with

intercalated crypt base columnar (CBC) stem cells (Figure from [35]).

### 3.3. Intestinal peptide and amino acid absorption

Ingested proteins are hydrolyzed into small peptides (length of 2-10 amino acids) by stomach and pancreas proteases and finally by brush-border membrane-bound peptidases[36]. The only absorbable units that may pass the BBM are tri- and dipeptides (absorption mediated by the luminal peptide transporter PEPT1) and single amino acids (AAs) (absorption mediated by various AA transporters expressed in the enterocytes' BBM (Figure 3))[37-40]. Within small intestinal enterocytes the absorbed tri- and dipeptides are hydrolyzed[41] and single AAs are released into the extracellular space by another set of AA transporters located in the basolateral enterocyte membrane (Figure 3) [1,37,38,40]. Interestingly, a similar set of transporters is used for small intestinal amino acid absorption as for amino acid re-absorption in proximal tubule kidney cells[1,36].

**Figure 3**

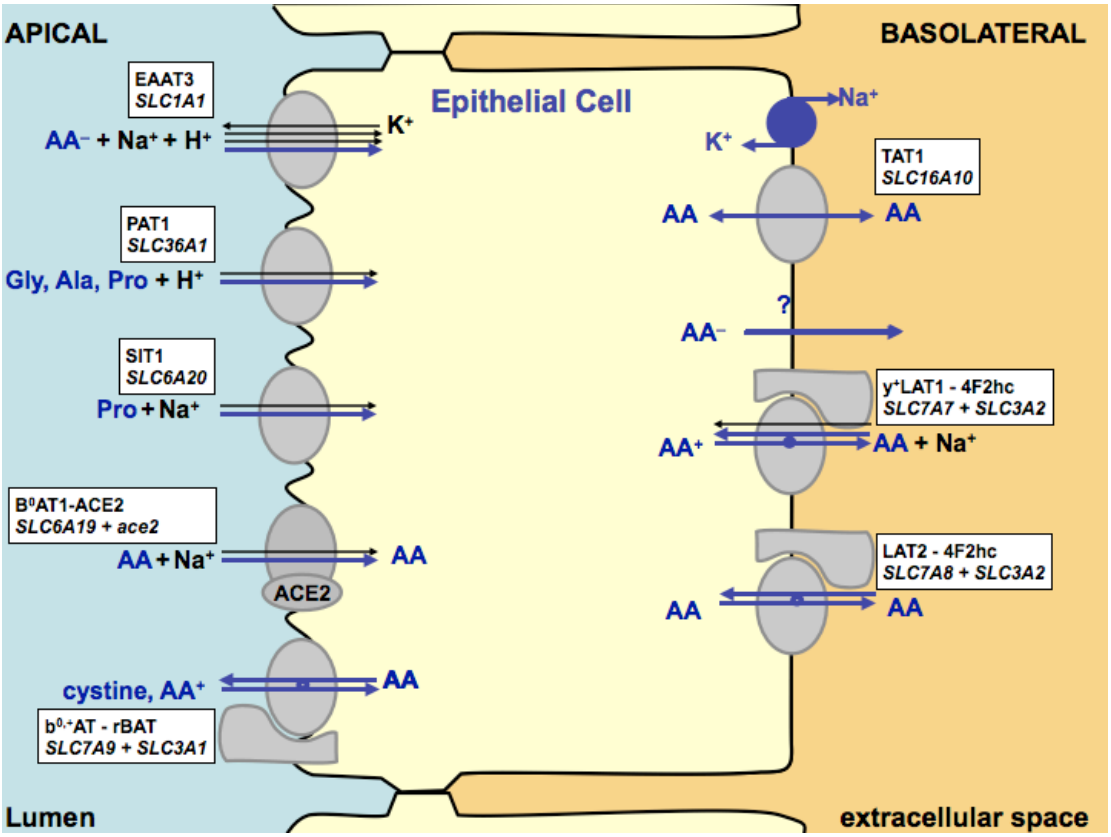


**Figure 3. Intestinal amino acid transport.** Scematic representation of amino acid transporters in a small intestinal enterocyte. The names of the transport proteins and their human genes are given. The small intestinal lumen is depicted in light blue (left side), enterocytes are given in yellow and the extracellular space is shown in orange (right side). Adapted figure from Verrey and co-workers[1].

### 3.3.1. SLC-families and nomenclature of (amino acid) transporters

Transport proteins may be categorized into active and passive transporters. Whereas the latter allow diffusion of solutes (as e.g. amino acids and monosaccharids) across cellular membranes according their electrochemical gradient, active transport systems are divided into primary- and secondary active systems[42]. While primary active transporters use the energy of ATP hydrolysis to drive molecules across membranes (as e.g. the Sodium-potassium ATPase; see Figure 3), secondary active transporters use transmembrane ion gradients (established by e.g. the basolateral Sodium-potassium ATPase) as driving force to transport solutes[42,43].

Since the 1990s membrane transporters in the human genome are grouped into solute carrier (SLC) families according to their sequence similarities (with transport proteins assigned to the same family sharing 20% or more amino acid sequence identity). Up to now, 52 (compared to initially 43 families) SLC families were identified including almost 400 human genes[42]. Thereof, 11 families include amino acid transporters[38].

Following the SLC nomenclature, human genes are named using the three letters *SLC*, followed by a digit (as e.g., *SLC6* designating the solute carrier family 6), followed by another letter defining the subfamily and another number identifying the individual transporter gene (as e.g., *SLC6A19*). Whereas human SLC transporter genes are written in upper-case letters (as e.g. SCL6A19), in rodent genes only the first letter is an upper-case character (as e.g. *Slc6a19*) and insect genes are designated using only lower-case letters[42].

### 3.3.2. Amino acid transporter $B^0AT1$

$B^0AT1$ (broad neutral amino acid transporter 1) (SLC6A19) is a sodium-dependent AA transporter expressed in the luminal enterocyte membrane and in kidney proximal tubule cells transporting neutral AAs[1,44] (with highest substrate affinity to large neutral amino acids, lower affinity to smaller neutral amino acids and lowest substrate affinity to amino acids L-glycine (Gly) and L-proline (Pro)[37].

Its defect causes *Hartnup disorder*, an autosomal recessive condition characterized by the urinary loss of neutral amino acids that is referred to as *neutral aminoaciduria*. First described in 1956[45] *Hartnup disorder* occurs at a frequency of about 1/30'000 in the European population and may cause clinical symptoms including pellagra-like light-sensitive rashes, diarrhea in infancies, cerebellar ataxia, and other central nervous system manifestations[4,5].

Efficient surface expression of $B^0AT1$ at the apical membrane of small intestinal enterocytes and kidney proximal tubule cells necessitates the co-expression of accessory proteins ACE2 (angiotensin converting enzyme 2)[5] and the type I membrane protein collectrin (TMEM27)[46], respectively. This interaction of $B^0AT1$ and collectrin (exhibiting about 40% sequence identity with the renin-angiotensin system (RAS) enzyme ACE2[47]) has been

discovered while studying the *collectrin* knock-out mouse which was showing an absence of $B^0AT1$ in the luminal proximal tubule cellular membrane that resulted in massive neutral aminoaciduria[46].

### 3.3.3. Amino acid transporter SIT1

The IMINO transporter SIT1 (sodium-dependent imino transporter 1; SLC6A20) designates a high affinity L-proline transporter, expressed – among other tissues – in the luminal membrane of small intestine enterocytes and proximal tubule kidney cells[48].
SLC6A20 mutations when occurring in combination with polymorphisms of other proline and glycine transporters may result in the metabolic disorder *Iminoglycinuria*, that is characterized by urinary loss of Pro and Gly and may be associated in some cases with hypertension, glycosuria, nephrolithiasis, mental retardation, deafness and blindness[49].
A functional interaction of SIT1 and ACE2 (in the small intestine) or collectrin (in the kidney), respectively, has been suggested by decreased intestinal L-proline absorption in *ACE2* knock-out mice and by reduced proximal tubule SIT1 expression and prolinuria in *collectrin* knock-out mice[46].

### 3.3.4. ACE2 – RAS enzyme, accessory protein of $B^0AT1$ and other functions

#### *3.3.4.1. ACE2 – RAS enzyme*

The systemic (or circulating) renin-angiotensin system regulates the blood pressure and controls the fluid and salt homeostasis[50]. Within the bloodstream, angiotensinogen (produced in the liver) is converted to angiotensin I by renin (produced by juxtaglomerular kidney cells following low blood pressure and/or low blood volume). Angiotensin I is then converted to angiotensin II by the angiotensin converting enzyme ACE, a zinc metalloendopeptidase that acts as a dipeptidyl carboxypeptidase on the surface of endothelial cells lining the blood vessel walls. Angiotensin II binds to the angiotensing II $AT_1$ (AGTR1) and angiotensin II $AT_2$ (AGTR2) receptors that mediate cellular effects including vasoconstriction, inflammation and cell proliferation (AGTR1) and aldosterone secretion from the adrenal cortex (Figure 5). This classic RAS has recently been extended by the function of the ACE homologue ACE2 which is a structural homologue to ACE and - at its carboxy-terminal domain - to the type I membrane protein collectrin[47] (Figure 4).

**Figure 4**

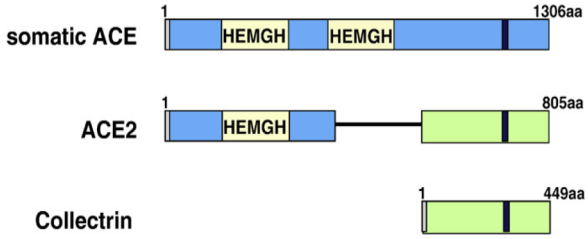


**Figure 4. Structures of ACE, ACE2 and collectrin.** The transmembrane domain is shown in black. The zinc-binding motif (HEMGH; carboxypeptidase) is repeated twice in ACE and once in ACE2 and is given in yellow. Regions of structural homology are given in the same color (from [47]).

ACE2 – similar to ACE – is a carboxypeptidase cleaving single C-terminal amino acids from angiotensin I and II thereby producing angiotensin 1-9 (Ang-(1-9)) and angiotensin 1-7 (Ang-(1-7)), respectively. The conversion of angiotensin II to Ang-(1-7) shows an over 400 times stronger efficacy than the Ang-(1-9) production and therefore definines the primary role of ACE2 within the RAS[50]. Ang-(1-7) binds to the MAS receptor, leading to vasodilation. ACE2 hence counteracts the RAS function by working against the AGTR1-mediated vasoconstriction and cellular proliferation[47,50,51] (Figure 5).

**Figure 5**

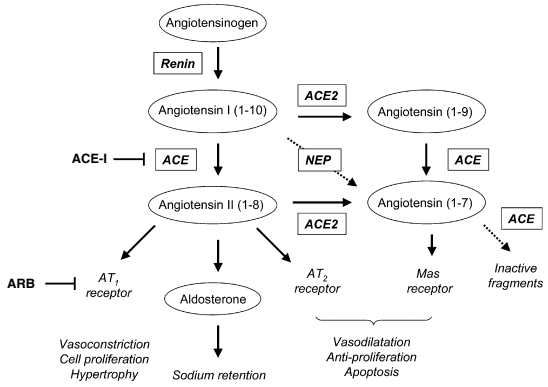


**Figure 5. The renin-angiotensin system.** ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; ACE-I, ACE inhibitor; ARB, angiotensin II $AT_1$ receptor blocker (modified from[52]).

#### *3.3.4.2. ACE2 – accessory protein of $B^0AT1$*

Brush-border membrane expression of $B^0AT1$ in small intestinal enterocytes depends on the presence of intestinal ACE2[5], similar as shown for collectrin in proximal tubule kidney cells[46]. Hence, *ACE2* knock-out mice showed an almost abolished BBM $B^0AT1$ expression

and $B^0AT1$-mediated AA transport into *Xenopus laevis* oocytes was increased upon co-expression of ACE2. In comparison to *collectrin* knock-out animals[46], kidney proximal tubule $B^0AT1$ expression was unchanged in *ACE2* knock out mice[5].

Whereas collectrin has no peptidase function, its functional homologue in small intestine hydrolyzes single C-terminal amino acids from small peptides and therefore fulfills two functions, trafficking $B^0AT1$ to the brush-border membrane and digesting nutrient peptides. These two processes were considered separately for many years, but seem to be linked together and are referred to as *digestive complexes* by Broer and co-workers[36] (Figure 6).

**Figure 6**

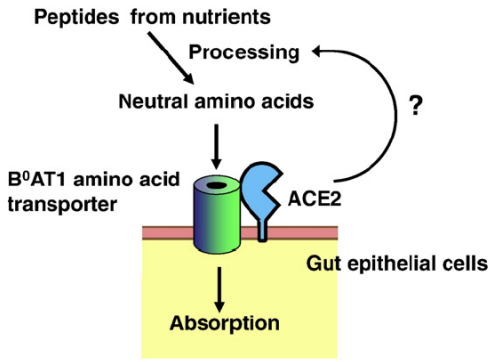


**Figure 6. $B^0AT1$ and ACE2 build a digestive complex.** ACE2 is required for luminal membrane $B^0AT1$ expression. Whether hydrolysis of luminal peptides via ACE2 also increases neutral amino acid supply for $B^0AT1$ was not known, when this figure was made and is therefore shown with a question mark (from [47]).

#### 3.3.4.3. *ACE2 – accessory protein of SIT1?*

A functional interaction of ACE2 and SIT1 has been suggested by the study of the *ACE2* knock out mouse model since sodium-dependent L-proline uptake in everted intestinal rings likely reflecting the secondary active transport via SIT1 was absent. A reduced small intestinal SIT1 expression in these mice could not been shown due to lack of a specific antibody[9].
On study showed that L-proline uptake in *X. laevis* oocytes expressing the mouse orthologue of SIT1 was not changed upon co-expression of ACE2, whereas co-expressing $B^0AT1$ and ACE2 increased transcellular L-leucine (Leu) transport[53].

#### 3.3.4.4. *Other ACE2 functions*

Non-peptide roles of ACE2 include being the functional receptor for the SARS coronavirus, a function that is attributed to the transmembrane domain of ACE2[47].

#### 3.3.4.5. *Regulation of ACE2 expression*

Whereas ACE-inhibitors (ACEIs) including captopril and lisinopril do not affect ACE2 activity[47], ACEIs and angiotensin II $AT_1$ receptor blockers (ARBs) increase the expression of ACE2 mRNA in different organs and tissues, including heart, kidney and the aorta[6-8]. A clear explanation for this observation could not been found yet[54].

## 3.4. Intestinal monosaccharide absorption

After digestion by the pancreatic α-amylase and brush-border membrane-bound disaccharidases including lactase, sucrase and maltase, dietary carbohydrates are absorbed exclusively (and unlike dietary proteins that may be absorbed as tri-, dipeptides and single amino acids) as monosaccharides D-glucose (Glu), D-galactose (Gal) and D-fructose (Frc)[16,55]. Three different intestinal transporters, SGLT1 (SLC5A1), GLUT2 (SLC2A2) and GLUT5 (SLC2A5) are well known to provide intestinal monosaccharide absorption[2,3]. SGLT1 is a high affinity, low capacity sodium-dependent brush border membrane (BBM) glucose and galactose transporter. GLUT2 and 5 are facilitated diffusion proteins with GLUT2 being expressed in the basolateral enterocyte membrane (but see also 3.3.1.), transporting Glu, Gal and Frc and GLUT5 transporting Frc across the BBM[15-17] (Figure 7).
More recently, another monosaccharide transporter predominantly localizing to the intestinal BBM, GLUT7 (SLC2A7), was identified, providing transmembrane glucose and fructose, but not galactose transport. Similarly as shown in rodents, this transporter seems also to be expressed in the human small intestine[56,57] (Figure 7).

### 3.4.1. Intestinal localization of monosaccharide transporter GLUT2

In experimental diabetes, but also in fructose- and/or glucose rich diets in rodents, the basolateral high-capacity, low-affinity facilitative glucose, fructose and galactose transporter GLUT2 also shows expression in the apical enterocyte membrane[18].
Of course small intestinal biopsies in humans are sampled during the fasting state (since the lumen has to be empty of food), a fact that might explain, that GLUT2 was detected only in the basolateral enterocyte membrane when human biopsies were examined for many years[18] until Ait-Omar et al. showed apical GLUT2 expression for the first time in morbidly obese human subjects in the year 2011[19]. This finding is in accordance with the fact that glucose absorption in humans rises at concentrations far above than required to saturate the high affinity, low capacity glucose and galactose transporter SGLT1, suggesting that high glucose diet leads to apical GLUT2 expression also in humans[18] (Figure 7).

**Figure 7**

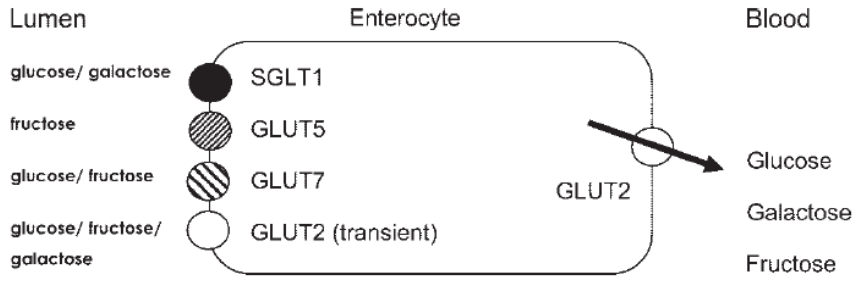


**Figure 7. Schematic representation of monosaccharide absorption in a small intestinal enterocyte** (from [57]).

### 3.4.2. Current definition of non-insulin-dependent diabetes mellitus

Non-insulin-dependent diabetes mellitus refers to a group of chronic metabolic diseases characterized by hyperglycemia, caused by a reduction of insulin secretion by pancreatic β-cells and/or peripheral (i.e. fat- and muscle tissue) insulin resistance[10]. According to the World Health Organization (WHO), since 1997 diabetes mellitus is defined as fasting plasma glucose levels ≥ 7.0 mmol/l (126 mg/dl) and/or plasma glucose levels ≥ 11.1 mmol/l (200 mg/dL) two hours after a 75 g oral glucose load as part of a glucose tolerance test[58]. Since 2009, glycosylated hemoglobin (HBA1c) levels with a cut-off point of ≥ 6.5% have been added to these diagnostic criteria[58,59].

### 3.4.3. Metformin treatment of non-insulin-dependent diabetes mellitus

The biguanide metformin is the most widely used drug to treat NIDDM[14,60] and was developed from galegine, a derivative of guanidine found in Galega officinalis (French lilac or goats rue) (Figure 8). According to the American Diabetes Association and European Association for the Study of Diabetes – if not contraindicated and if tolerated – metformin is the preferred first-line treatment for NIDDM[11]. The glucose-lowering effect of metformin is mainly attributed to decreased hepatic glucose output and increased peripheral glucose uptake. Other mechanisms that may contribute are reduced fatty-acid oxidation and increased intestinal use of glucose[12,13]. Furthermore, metformin treatment exerts an inhibitory effect on intestinal glucose absorption as shown in rodents[14] but inconsistent effects of metformin on intestinal monosaccharide transporter (SGLT1, GLUT2 and GLUT5) expression were found in non-diabetic rats[61,62].

**Figure 8**

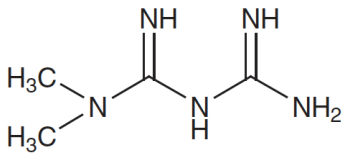


**Figure 8. Chemical structure of metformin**[60].

### 3.4.4. Effects of dietary carbohydrates and/or (experimental) diabetes on intestinal monosaccharide transporter gene expression

Several studies show dietary effects on intestinal monosaccharide transporter expression. Dietary fructose and sucrose increased jejunal hexose transporter (including SGLT1, GLUT2 and GLUT5) transcript expression in non-diabetic rats[17]. Whereas expression of the fructose transporter GLUT5 seems to be specifically responsive to fructose, intestinal expression of SGLT1 and GLUT2 also depend on dietary glucose[63]. The regulation of intestinal hexose transporters in diabetic subjects seems to be complex and elicits contradictory findings: Whereas one study in humans shows increased gene and protein expression of intestinal hexose transporters GLUT2, GLUT5 and SGLT1 in humans with NIDDM[16], another study performed in Zucker rats – reflecting a model of type 2 diabetes – shows no effect on intestinal monosaccharide transporter GLUT2, GLUT5 and SGLT1 expression[20]. Surveys in rodents with streptozotocin-induced type 1 diabetes show increased small intestinal GLUT2 and SGLT1 expression[64,65] with inconsistent findings concerning small intestinal GLUT5 expression[63,65].

## 3.5. Parkinson's disease and intestinal levodopa absorption

### 3.5.1. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder (following Alzheimer's disease). It is caused by cell loss in different brain regions, mainly by damage to dopaminergic neurons in the substantia nigra[66] (Figure 9a). Histo-morphologically, PD is characterized by the presence of *Lewy bodies*, representing eosinophilic cytoplasmic inclusions of fibrillar structure showing a dense core, surrounded by a halo (Figure 9b).

**Figure 9a**

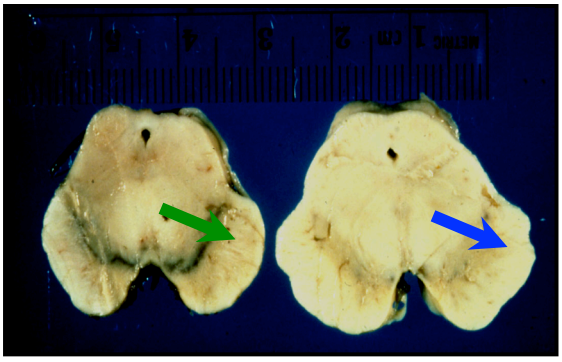

**Figure 9b**

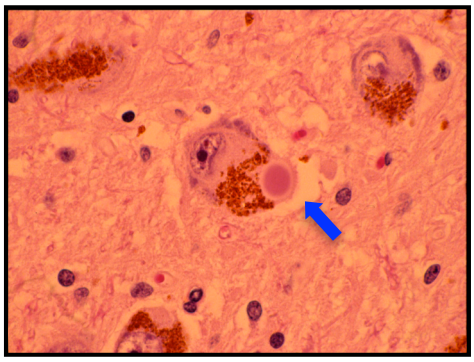

**Figure 9a. Substantia nigra.** The substantia nigra from a control patient (left side; green arrow) and from a Parkinson's disease patient (right side; blue arrow) are shown. In parkinson's disease, the substantia nigra is almost totally pallid due to loss of pigmented neurons. **Figure 9b. Lewy body.** An eosinophilic *Lewy body* surrounded by a slight halo (blue arrow) within a pathologic neuron is shown. Pictures were adapted from a power point presentation from James B. Leverenz, MD, Associate Professor Neurology and Psychiatry and Behavioral Sciences, University of Washington School of Medicine.

PD typically manifests above the age of 60 years and displays characteristic clinical symptoms including bradykinesia, resting tremor and rigidity[21]. At its onset, PD patients show slowly starting and often misinterpreted symptoms of fatigue and stiffness. A lugubrious stiff face and postural changes may follow[21] (Figure 10).

**Figure 10**

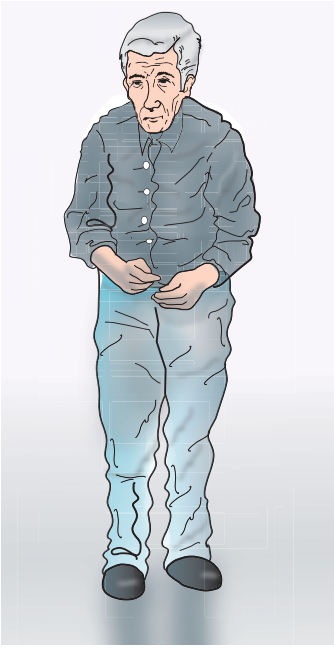

**Figure 10. Illustration of a typical Parkinson's disease patient** showing a frozen face and characteristic postural changes (from [21]).

### 3.5.2. Levodopa – the main therapeutic agent in Parkinson's disease

First introduced in 1968, the dopamine precursor levodopa (L-Dihydroxyphenylalanine, L-Dopa) is still the main therapeutics for the treatment of Parkinson's Disease[22]. After passing the Blood Brain Barrier (BBB) via the L-type amino acid transporter 1 (LAT1)/4F2 heavy chain (4F2hc) complex[67], levodopa is converted into dopamine by the dopa-decarbocylase[23]. To prevent levodopa metabolism prior to its transport across the BBB, orally administered levodopa is routinely given in combination with a DDC inhibitor (DDCI) (as e. g. carbidopa or benserazide) that may be combined with a COMT inhibitor (such as entacapone) in addition to avoid levodopa methylation into 3-0-methyldopa[22].

The clinical response to levodopa therapy may be categorized into short- and long-term response. Whereas the short-duration response seems to be preserved during disease progression, the long-term response progressively deteriorates, clinically manifesting in daily fluctuating motor responses, referred to as *'on-off' phenomenon*[23,68]. This phenomenon has been attributed to an unsteady delivery of levodopa to the dopamine-lacking substantia nigra together with the inability to compensate in end-stage disease with depleted dopamine stores[68].

### 3.5.3. Small intestinal levodopa absorption

Levodopa is a large neutral amino acid (LNAA) that shows structural homology to aromatic amino acids (AAs) L-phenylalanine (Phe) and L-tyrosine (Tyr). Therefore, levodopa has been suggested to being absorbed by neutral amino acid transporters in the small intestine[69]. Hence, as shown in 2000, levodopa is transported via the cysteine and dibasic AA exchanger $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1)[24] that is expressed in the luminal enterocyte membrane. $b^{0,+}$AT is the catalytic unit, that is covalently linked to the glycoprotein rBAT forming a heteromeric AA exchanger[70]. Whereas at the luminal enterocyte membrane only one transporter ($b^{0,+}$AT-rBAT) was identified transporting levodopa, three different AA transporters were shown to carry levodopa across the basolateral membrane, namely the $Na^+$-independent L-type amino acid exchangers LAT1 (SLC7A5) and LAT2 (SLC7A8)[25], as well as the aromatic AA uniporter TAT1 (SLC16A10)[26]. Similarly as $b^{0,+}$AT and the heavy chain rBAT, also the light chains LAT1 and LAT2 (that form the transmembrane domains) are covalently linked to a glycosylated type II membrane protein, the heavy chain 4F2hc (SLC3A2) that traffics and stabilizes the associated light chains[70]. In addition to $b^{0,+}$AT-rBAT, other luminal AA transporters including the broad neutral amino acid transporter $B^0$AT1 have been proposed to transport levodopa[24].

In patients with end-stage parkinsonism and fluctuating motor response to levodopa treatment, it has been hypothesized, that dietary proteins may affect small intestinal levodopa absorption due to competition of levodopa with dietary amino acids for its transport across the brush border membrane[68].

# 4. Aims

Several factors affect small intestinal SLC transporter expression and function, including diet, (gastrointestinal) diseases, medications, circadian rhythm, functional interactions among different transporters and the presence of accessory proteins. The aim of this PhD work was to assess the effect of certain medications (ACE-inhibitors, angiotensin II $AT_1$ receptor blockers and metformin), diseases (type 2 diabetes mellitus), and of transporters' substrates (levodopa, glucose, amino acids) on amino acid and monosaccharide transporters expression in human small intestine.

In a first study we aimed to assess the effect of antihypertensive drugs (ACE-inhibitors and angiotensin II $AT_1$ receptor blockers) on small intestinal ACE2 and associated transporters ($B^0AT1$, etc.) expression. Furthermore, a putative functional interaction of ACE2 and system IMINO transporter SIT1 was investigated.

In a second project, we aimed to test whether type 2 diabetes mellitus and/or metformin treatment affects small intestinal monosaccharide transporter (GLUT2, GLUT5 and SGLT1) expression. In addition, the putative luminal expression of basolateral GLUT2 in non-treated diabetic patients was assessed (results pending when the present report was written).

In a third project, we studied the intestinal absorption mechanism of the amino acid and main Parkinson's disease therapeutical levodopa. Because different AAs may compete for its intestinal absorption, we further tested the effect of routinely co-administered carbidopa and bensirazide and of dietary amino acids on intestinal levodopa absorption.

# 5. Manuscript: *'Human intestine luminal ACE2 and amino acid transporters are regulated by ACE-inhibitors'*

This section contains the manuscript entitled '*Human intestine luminal ACE2 and amino acid transporters are regulated by ACE-inhibitors'* that was sent to the *American Journal of Physiology – Gastrointestinal and Liver Physioloy* the 12th of December 2013 and returned for further correction.

***Human intestine luminal ACE2 and amino acid transporters are regulated by ACE-inhibitors***

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***Running Head*** Human intestine ACE2 and amino acid transporters

***Contributors***

RNV and FV were responsible for study design, data analysis and writing of the paper; RNV, SMC, OG, MFo, WS, MFr, and FV planned the study. Patient recruitment was performed by RNV, LE, TS, ZFK, SK, and OG. Experimental procedures were conducted by RNV, LE, TS, EK, JJ, QMH and SH. Promoter module analysis was performed by ML.

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***Word count***

5137 (Introduction, Materials & Methods, Results and Discussion)

*Annotation: The reference numbers within this manuscript refer to the references listed in chapter 10.*

***Abstract (248 words)***

Sodium-dependent neutral amino acid transporter $B^0AT1$ (SLC6A19) and imino acid (proline) transporter SIT1 (SLC6A20) are expressed at the luminal membrane of small intestine enterocytes and proximal tubule kidney cells where they exert key functions for amino acid (re)absorption as documented by their role in Hartnup disorder and iminoglycinuria, respectively. Expression of $B^0AT1$ was shown in rodent intestine to depend on the presence of the carboxypeptidase angiotensin converting enzyme 2 (ACE2). This enzyme belongs to the renin-angiotensin system and its expression is induced by treatment with ACE-inhibitors (ACEIs) or angiotensin II $AT_1$ receptor blockers (ARBs) in many rodent tissues. We show here in the *Xenopus laevis* oocytes expression system that human ACE2 also functionally interacts with SIT1. To investigate in human intestine the potential effect of ACEIs or ARBs on ACE2, we analyzed intestinal biopsies taken during routine gastroduodenoscopy and ileocolonoscopy from 46 patients of which 9 were under ACEI and 13 ARB treatment. Analysis of transcript expression by real-time PCR and of proteins by immunofluorescence showed a co-localization of SIT1 and $B^0AT1$ with ACE2 in the brush-border membrane of human small intestine enterocytes and a distinct axial expression pattern of the tested gene products along the intestine. Patients treated with ACEIs displayed in comparison with untreated controls increased intestinal mRNA levels of ACE2, peptide transporter PEPT1 (SLC15A1) and AA transporters $B^0AT1$ and PAT1 (SLC36A1). This study unravels in human intestine the localization and distribution of intestinal transporters involved in amino acid absorption and suggests their regulation by ACEIs.

***Keywords***

$B^0AT1$; SIT1; ACE2, angiotensin converting enzyme inhibitors; intestine

**INTRODUCTION**

Protein digestion refers to the hydrolysis of dietary proteins by stomach and pancreas proteases and by brush-border membrane-bound peptidases into absorbable units, namely tri-, dipeptides and amino acids (AA) [39]. Transepithelial absorption across enterocytes involves then sequential transport across the luminal brush-border and basolateral membrane. The luminal step is mediated by the peptide transporter PEPT1 and various AA transporters [37-40]. From enterocytes, amino acids are then released into the extracellular space by another set of AA transporters located in the basolateral membrane [41,71].

The major small intestine luminal transporter for neutral AAs $B^0AT1$ (broad neutral AA transporter 1, SLC6A19) is also expressed in kidney proximal tubule and its defect was shown to cause Hartnup disorder [72,73]. Intestinal $B^0AT1$ expression and function depends on the presence of the accessory protein ACE2 (angiotensin converting enzyme 2) [5,53]. This membrane-anchored monocarboxypeptidase is a structural homologue of ACE and is expressed in various tissues, including heart, kidney, testes, lung and intestine, where it negatively regulates the renin-angiotensin system by degrading angiotensin I and II into Ang-(1-9) and Ang-(1-7), respectively [47]. At the brush-border membrane of small intestine enterocytes, ACE2 displays a high expression level and is suggested to participate to peptide digestion [53,74]. The catalytic domain of ACE2 is fused to a membrane anchor domain that shows high structural similarity with the renal protein collectrin (TMEM27) [47] which interestingly functions as $B^0AT1$-associated protein in kidney proximal tubule [46].

The sodium-dependent imino transporter 1 (SIT1,SLC6A20) is a high affinity luminal L-proline (Pro) transporter, expressed – among other tissues – in small intestine enterocytes and proximal kidney tubule cells [48,75]. Mutations of its gene has been suggested to cause – in combination with polymorphisms of other proline and glycine transporters – the metabolic disorder Iminoglycinuria [49]. SIT1 (SLC6A20) is structurally closely related to $B^0AT1$ (SLC6A19) but an analogous functional interaction with ACE2 and/or collectrin has not been demonstrated, although suggested by the observation that mice lacking the ACE2-related protein collectrin display reduced proximal tubule SIT1 expression and significant prolinuria [46]. Similarly, *ACE2* knock-out mice show decreased intestinal Pro absorption; however, effects on SIT1 protein expression could not be tested due to lack of a specific antibody [9].

Drugs interfering with the renin angiotensin system (RAS), such as ACEIs and ARBs have become first-line medications to treat arterial hypertension [76]. Interestingly, both types of drugs have been shown in rodents to increase the expression of ACE2 mRNA in different organs and tissues, including heart, kidney and the aorta [6-8]. However, it is not known, whether ACEIs or ARBs also affect the expression of small intestine ACE2, which is involved – as a carboxypeptidase – in protein digestion and – by interacting with $B^0AT1$ and potentially SIT1 – in AA absorption. Additionally, the knowledge about the axial distribution of AA- and peptide transporters along the intestine is sparse and mainly originates from animal studies [70,77].

In this study we (i) addressed the question whether human ACE2 can interact functionally with the proline transporter SIT1 using the *Xenopus laevis* oocyte expression system and (ii) whether it co-localizes with SIT1 as with $B^0AT1$ at the luminal surface of human intestinal mucosa. Furthermore, we (iii) analyzed the axial distribution of ACE, ACE2, amino acid- and peptide transporters in human intestine and (iv) assessed whether ACEIs and ARBs potentially impact on ACE2 and AA transporter expression in the intestine of human patients.

## MATERIALS AND METHODS

### Transport Studies in *Xenopus laevis* oocytes

Transport studies using radiolabeled amino acid tracers were performed as described previously [78]. Briefly, after cRNA injection (SIT1 alone: 5ng, SIT1 and ACE2: 5ng and 20ng, SIT1 and collectrin: 5ng and 5.4ng) oocytes were incubated 2 – 3 days in ND96 solution at 16°C. Thereafter, 6 – 10 oocytes per condition were washed 3 - 4 times at room temperature (RT) with uptake buffer (pH 7.4, 10mM HEPES, 100mM NaCl, 2mM KCl, 1mM $MgCl_2$, and 1mM $CaCl_2$) and pre-incubated at 25°C for 2 minutes. The uptake solution containing radiolabeled amino acids (2µCi of radiolabeled tracer per group of oocytes) was added for 10 minutes and uptake was ended by washing oocytes 4 times with uptake buffer at 4°C. Oocytes were then dissolved separately in SDS (2%) for 60 minutes. Finally, 3ml of scintillation solution (Emulsifier-Safe TM) was added to each oocyte and radioactivity was determined using a scintillation counter (TRI-CARB 2900TR, Packard Instrument Co., Meriden, CT). Data were expressed as pmol/h/oocyte, and values obtained for non-injected oocytes were subtracted. Kinetic experiments were performed with five different amino acid concentrations ranging from 3.3 to 330µM.

### Study population

A total number of 46 (21 male and 25 female) patients was included in the current study, with 9 (20%) patients treated with ACE-Inhibitors, 13 (28%) patients treated with $AT_1$-receptor blockers and 24 control patients (Table 1). Medical supply was independent from the present study or hereby obtained results. 24 (52%) control patients were not on medication that affected the Renin-Angiotensin system. All patients were examined at one single institution. Patients underwent either gastroduodenoscopy only (n = 34), a combined gastroduodenoscopy and ileocolonoscopy (n = 10) or ileocolonoscopy only (n = 2) as part of a routine medical checkup. During this procedure, mucosal biopsies were taken at four different parts of the gastrointestinal tract: Duodenum parts II (descending) and III (inferior/horizontal), terminal ileum and ascending colon. Blood and urine samples were collected from all patients in order to quantify amino acid levels using HPLC (High Performance Liquid Chromatography) measurements. Laboratory values of the RAS (ACE-activity, renin, aldosterone, angiotensin I and II; measured at the Institute of Clinical Chemistry, University Hospital of Zurich), as well as different physiologic parameters, such as body mass index (BMI), mean arterial blood pressure (MAP = (systolic blood pressure + 2x diastolic blood pressure) /3) and heart rate were assessed. The age of included patients ranged from 18 to 80 years and patients BMI was between 18 and 35kg/$m^2$. Patients with severe pathologies of the gastrointestinal tract, such as coeliac disease, Crohn's disease and ulcerative colitis, as well as patients with carcinomas, kidney- or hepatic insufficiency, bleeding disorders, infectious diseases, oral anticoagulation, drug- or alcohol abuse or mental retardation, were excluded from the present study.

Mean age of treated patients (65 years) was higher than of non-treated controls (52 years). Body mass index (BMI), mean arterial pressure, angiotensin I and II and renin plasma levels were not different between groups. Plasma ACE-activity was significantly lower in patients treated with ACEIs when compared to non-treated controls (Table 1).

**Intestinal biopsies**

Mucosal biopsies were taken at four different intestinal localizations (duodenum part II, part III, terminal ileum and ascending colon). After removal, tissue specimens were immediately frozen in liquid nitrogen and stored at -80$^0$C until RNA extraction or cryosection was performed.

**Immunofluorescence**

Cryosection

Harvested biopsies were embedded in optical coherence tomography (OCT) cryostat medium (Medite Medizinaltechnik AG, Switzerland) and put into tubes containing liquid propane. Tubes were transferred into liquid nitrogen for quick freezing. Obtained blocks were stored at -80°C until further processing. A cryotome, (Leica CM 1850 Cryostat, Switzerland) was used to produce 5µm sections that were immediately transferred onto polylysine slides (O. Kindler & CO GmBH, Germany). The slides were stored at -20°C until later processing.

Immunostaining

Immunostaining of frozen tissue sections was performed as described previously [79]. Briefly, samples were defrosted at RT for 5 minutes in a wet chamber. For fixation, slides were immersed in Methanol (70%) for 90 seconds. After fixation, slides were washed (1 x 15min, 2 x 5min) with phosphate buffered saline (PBS, 0.1M) (137.0mM NaCl, 2.7mM KCl, 12.0mM $HPO_4^{2-}/H_2PO_4^-$). To reduce unspecific antibody binding, tissues were kept in 2% bovine serum albumin (BSA) diluted in PBS (0.1M) for 1h at RT. The following primary antibodies were applied to the section samples for 1 hour at RT: 1. Affinity purified mouse anti human polyclonal SIT1 (Abnova, Taipei, Taiwan), 2. affinity purified goat anti human polyclonal ACE2 (R&D Systems, Minneapolis, USA), 3. affinity purified rabbit anti human polyclonal B$^0$AT1 (Pineda, Berlin, Germany). Antibodies were diluted 1:100 in PBS (0.1M) enriched with 2% BSA and 0.04% Triton X-100. Thereafter, sections were rinsed again in PBS (0.1M) (3 x 5min) before incubation with the secondary antibodies (Alexa Fluor® 594 donkey anti-mouse IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-mouse IgG, dilution 1:500, Alexa Fluor® 594 donkey anti-goat IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-goat IgG, dilution 1:500, Alexa Fluor® 594 donkey anti-rabbit IgG, dilution 1:500, Alexa Fluor® 488 donkey anti-rabbit IgG, dilution 1:500) and 4', 6'-diamidino-2-phenyl- indole (DAPI, Merck, NJ) (0.1mg/ml) (diluted 1:5'000) for 1 hour at RT. Sections were washed with PBS (0.1M) (3 x 5min) before mounting with DAKO fluorescence mounting media (DakoCytomation, Baar, Switzerland). Sections were examined using a Nikon Eclipse TE300/200 inverted microscope fitted with a

DS-5M Standard charge-coupled device camera. Pictures were captured with NIS-Elements software (Nikon Instruments Inc, Melville, NY) and processed using Adobe Photoshop software. Incubation of sections with secondary antibodies only did not result in a detectable signal (data not shown).

**Intestinal gene expression**

RNA extraction

RNA extraction was performed by disrupting tissue in 350µl RLT-Beta-Mercapto-Ethanol buffer (10µl β-ME / 1ml RLT-buffer) with MagNALyser Green Beads (Roche, Switzerland) for 30 seconds at 6000rpm using a Precellys® 24 tissue homogenizer (Bertin Technologies, France). The solution was centrifuged at 10'000g for 5 minutes at 10°C. The supernatants were directly used for RNA isolation with the QiagenRNeasy mini kit (QIAGEN, Switzerland), which was employed according to the manufacturer's instructions.

RNA concentrations were determined using the NanoDrop 1000 Spectrophotometer (Witec AG, Switzerland) at 260nm wavelength. Beside RNA concentrations, purity of the extractions was assessed.

Reverse transcription

For reverse transcription, Applied Biosystems Taq Man RT-PCR reagents and the Biometra T Gradient Thermocycler (Biolabo Scientific Instruments SA, Switzerland) were used. Final concentrations in the reaction mix were: RT buffer (1x), $MgCl_2$ (5mM), random hexamers (2.5µM), deoxyNTP mix (500µM each), RNAse inhibitor (0.4Ux$\mu l^{-1}$), multiscribe reverse trancriptase enzyme (1.25Ux$\mu l^{-1}$), RNA template (33ngx$\mu l^{-1}$) and RNAse free water. All reactions were executed with negative controls (RT-) using the same protocol without adding the multiscribe reverse trancriptase enzyme to the reaction mix. Until further analysis, samples were stored at -20°C.

Primers and probes

Primers and probes were designed according to a previous report [80] or using the software Primer Express 3.0 (Applied Biosystems, Switzerland). Self-designed primers were chosen to generate amplified fragments of 70 to 140 base pair length spanning intron-exon boundaries to avoid contaminating genomic DNA. Primer specificity of all primers was tested using mRNA from human intestine and/or human kidney and resulted in a single product of expected length (data not shown). Probes were labelled with the fluorescent reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth AG, Switzerland) (Supplementary Table 1).

Real time PCR

Quantitative Real Time PCR (qRT-PCR) was performed as described previously [70,79]. Briefly, a 20µl PCR reaction volume was prepared using cDNA (1µl), TaqMan Universal PCR

Master Mix (10µl) (Applied Biosystems AG, Switzerland), Primers (0.8µl each), Probe (0.4µl) and DEPC-Water (7µl). Final concentrations in the reaction volume were: Primers (1µM each) and Probe (0.1µM). Reactions were run in 96-well optical reaction plates using the 7500 Fast Real-Time System Termocycler (Applied Biosystmes AG, Switzerland). 45 thermal cycles were set at 95°C (10 minutes), 60°C (15 seconds) and 72°C (1 minute). For analyzing data, an individual threshold was set for each gene in the linear range of the amplicon curves. All reactions were run in triplicates and mean values were used for further processing. Negative controls (RT-) were run for each gene. If maximal cycle difference within triplicates was ≥ 1 cycle or if the difference between the mean value of the triplicates and the negative control was ≤ 5 cycles, obtained results were discarded and not used for further analysis. Abundance of target mRNA was calculated relative to a Villin mRNA, which is commonly used as reference gene for epithelial content in human intestinal samples [81]. In order to verify non-varying Villin mRNA expression along the human digestive tract, target and Villin mRNA was calculated relative to a second reference gene (HPRT, encoding the Hypoxanthine phosphoribosyltransferase) (data not shown) [82]. Relative gene expression values were determined using the ΔCt method (relative expression = $2^{-\Delta Ct}$, ΔCt = average Ct value of target – average Ct value of reference).

**Comparative promoter analysis**

The proximal promoter regions of human ACE2, $B^0AT1$ and PEPT1 genes were retrieved using the software Gene2Promoter (Genomatix). The proximal promoter regions used were generally defined as 500 nucleotides upstream and 100 nucleotides downstream from the transcription start site (TSS). TSSs were automatically assigned to genes based on 5′ cap site databases integrated into promoter identification programs (Eldorado, Genomatix). Promoter sequences of relevant transcripts and in a second analysis of relevant transcripts plus CompGene promoters (promoters with no transcript listed) were included in the analysis. Genomatix FrameWorker database was used to identify new TFBS modules [83]. A module is defined as a set of two or more TFBSs with a specific order, strand orientation, and distance range between the individual TFBSs. A threshold of 100% genes containing the module, a distance of 5 – 200 bp and an intersite variability of 15 bp or less were permitted between TFBSs. The software ModelInspector (Genomatix) was used with the default settings to identify the previously characterized TF binding modules in additional promoter regions of the Genomatix Human Promoter Database (Version Eldorado 12-2012) [84].

**Statistical analysis**

For data representation and statistical analysis, the statistical software Graphpad Prism 5 (GraphPad Software, San Diego, CA) and **R, an open-source language and environment for statistical computing. (** *http://www.R-project.org/***)** was used. Shown error bars correspond to standard error of the mean (SEM). *p*-values of 0.05 or less were considered statistically significant.

**Ethics**

Human experiments

Written informed consent was obtained from each patient. The study was conducted according to Good clinical practice guidelines and was approved by the local ethics committee (reference number: EK-1744).

Animal experiments

Animal experiments (i.e. removal of oocytes from *Xenopus laevis* frogs) were performed according to the Swiss Animal Welfare Laws and as approved by the local Veterinary Authority (Kantonales Veterinäramt Zürich).

## RESULTS

### Functional interaction of human L-proline transporter SIT1 with ACE2 and collectrin in *Xenopus laevis* oocytes

To test whether human ACE2 and human collectrin functionally interact with SIT1 – as shown for $B^0AT1$ [53] – we co-expressed these proteins in *Xenopus laevis* oocytes and assayed their transport function using L-proline as substrate. Pro uptake rate was increased more than 2-fold, when ACE2 or collectrin were co-expressed with SIT1, whereas no significant Pro uptake was observed when ACE2 or collectrin RNA was injected alone (Figure 1A). To test whether these two accessory proteins modulate SIT1 transport kinetics differentially, the concentration dependence of L-proline uptake was measured. The apparent affinity of SIT1 alone ($K_{0.5}$: 40.5 µM) was similar in oocytes co-expressing ACE2 ($K_{0.5}$: 47.9 µM) or collectrin ($K_{0.5}$: 42.1 µM), respectively, as shown by the $K_{0.5}$ derived from Michaelis-Menten curves fitted to the experimental points. In contrast, the maximal transport capacity ($V_{max}$) was about 3-fold higher in oocytes co-injected with ACE2 or collectrin, respectively (Figure 1B).

### Localization of amino acid transporters $B^0AT1$ and SIT1 and of ACE2 in human small intestine

The distribution of the AA transporters $B^0AT1$ (Figure 2A; green) and SIT1 (Figure 2D; green) and of ACE2 (Figure 2B and E; green) along the villi (V) and in the crypts (C) was visualized by immunohistochemistry. The two transporters co-localized with ACE2 along the brush-border membrane of duodenum (Figures 2C and F) and terminal ileum (not shown) enterocytes on villi. The signal of AA transporter $B^0AT1$ (Figure 2A) appeared to be stronger towards the tips of the villi and weaker in the crypts, whereas no clear statement about the expression along the crypt-to-villus axis of SIT1 and ACE2, respectively may be made based on the present pictures. Some cells lining the small intestine lumen showed a diffuse labeling with all antibodies and represent presumably goblet cells that are non-specifically stained. Whereas in the colon $B^0AT1$ was not detected by immunofluorescence, ACE2 and SIT1 were labeled within colonic crypts where they co-localized to the apical membrane of epithelial cells (data not shown).

### Axial expression of AA- and peptide transporters as well as of ACE and ACE2 mRNAs along human intestine

Using mucosal biopsies taken from 10 patients referred for combined gastroduodenoscopy and ileocolonoscopy, real-time PCR was performed to measure the relative abundance of SLC transporter mRNAs in the epithelial layer of duodenum parts II and III, terminal ileum and ascending colon. As internal standard, the transcript of the small intestine enterocyte housekeeping gene Villin was used [81]. No statistical tests were applied to the transporters' distribution: terms like *higher, lower* and *equal* only qualitatively describe the data and do not

refer to statistical *significant differences.* These measurements revealed distinct axial gene expression patterns (Figure 3): (i) All tested transporter mRNAs were more abundant in small than large intestine with the exception of that of the AA antiporters ASCT2 (SLC1A5) and $y^+$LAT2 (SLC7A6) (catalytic subunit of 4F2hc (SLC3A2)) that displayed a higher gene expression in the ascending colon. (ii) The luminal transporter mRNAs showed equal expression levels along the duodenum and terminal ileum except the one of the cationic AA and cystine exchanger subunit $b^{0,+}$AT (SLC7A9) that was higher in the terminal ileum (Figure 3A). (iii) Basolateral transporter subunit mRNAs of LAT1 (SLC7A5), $y^+$LAT1 (SLC7A7) and $y^+$LAT2 were equally expressed along the duodenum and terminal ileum, whereas the mRNAs of the transporters/transporter subunits LAT2 (SLC7A8), 4F2hc (SLC3A2), LAT4 (SLC43A2) and TAT1 (SLC16A10), showed a lower gene expression level in the distal ileum than in the duodenum (Figure 3B). (iv) The mRNA encoding ACE was higher in the terminal ileum, whereas that of ACE2 was equal along the small intestine (Figure 3A).

**Intestinal gene expression of ACE2, $B^0$AT1, SIT1, PAT1 and PEPT1 in patients treated with ACEIs**

Mucosal biopsies from 42 patients referred for gastroduodenoscopy were analyzed. Nine of these patients were treated with ACE-Inhibitors, 13 with $AT_1$-receptor blockers, 22 had no RAS-active treatment and none was treated with both medications.

Levels of mRNA expression of all gene products assessed were very similar in duodenum parts II and III, whereas clear differences between duodenum and terminal ileum and between small and large intestine were observed for several genes (Figure 3). The impact of ACEI and ARB treatment on mucosal gene expression was assessed for duodenal samples (means of all available biopsies). In patients treated with ACEIs the mean duodenal mRNA expression level of ACE2 was increased 1.9-fold when compared to non-treated controls and that of the SLC transporters $B^0$AT1, PEPT1 and PAT1 1.7-, 1.6- and 1.6-fold, respectively. No significant differences in ACE2, $B^0$AT1, PEPT1 or SIT1 expression levels were observed in patients treated with ARBs (Figure 4A). None of the other genes assessed, including SIT1, showed significant differences between groups (data of the other genes not shown).

To test whether the ACEI-induced regulation of intestinal ACE2, $B^0AT1$, PAT1 and PEPT1 expression are correlated with each other, we determined the Pearson correlation of respective mean duodenal mRNA levels in all patients (n = 44) quantifying the strength of the linear association. Gene expression of intestinal ACE2 strongly correlated with that of $B^0AT1$ (correlation coefficient 0.83, confidence interval 0.70 – 0.90, $p < 0.0001$), and PEPT1 (correlation coefficient 0.80, confidence interval 0.63 – 0.89, $p < 0.0001$). The correlation of ACE2 with PAT1 (correlation coefficient 0.34, confidence interval 0.02 – 0.60, $p < 0.05$) is quite small and only weakly significant (Figure 4B-D).

**Promoter analysis**

As the mRNA expression of ACE2, $B^0AT1$, and PEPT1 correlated with each other, we performed a comparative promoter analysis to identify common regulatory sequences. Comparison of the proximal promoter regions of relevant transcripts of these three genes revealed a common promoter module with three conserved transcription factor binding sites (TFBS) (SORY_EBOX_MIZ1_modules). Screening of the human promoter database for the occurrence of this module revealed its presence only in six other genes, namely HUS1, HIP1, CASR, GALC, ZNF555, and VPS13C. A second analysis including all potential promoter regions of ACE2, $B^0AT1$, and PEPT1 detected a single additional module with five conserved TFBS (HOXC_FKHD_HOMF_HOMF_RORA_module) that was found in a single other gene (CDV3). The expression and potential co-regulation of these genes sharing promoter modules with ACE2, $B^0AT1$ and PEPT1 was tested in the small intestine biopsies. Only four of these seven genes displayed an mRNA expression level reliably quantifiable by qPCR and none of them was increased in ACI-treated patients (data not shown), indicating that the effect described above for ACE2, $B^0AT1$ and PEPT1 transcripts is selective for these luminal membrane proteins.

## DISCUSSION

The ACE2 carboxypeptidase is an important player of the systemic RAS and is also expressed at the surface of the small intestine [5,53]. In enterocytes, ACE2 is required for the expression of the major neutral amino acid transporter $B^0AT1$ the defect of which causes Hartnup disorder. A similar interaction with ACE2 has been proposed for the L-proline transporter SIT1 the defects of which contributes to iminoglycinuria [49]. The deficiency of ACE2 impairs amino acid absorption in the mouse, in particular of tryptophan, and thereby increases susceptibility to intestinal inflammation [85]. In rodent heart, kidney and aorta, treatment with ACEIs and/or ARBs was shown to increase ACE2 expression [6-8].

This study addressed the question of the role of ACE2 in human intestine. First we characterized its functional interaction with the proline transporter SIT1 in *Xenopus laevis* oocytes. Then we demonstrated the axial expression of ACE2 along human intestine and also that of ACE, AA- and peptide transporters. Finally we showed that patients treated with ACE-inhibitors express higher transcript levels of intestinal ACE2, amino acid transporters $B^0AT1$ and PAT1 and peptide transporter PEPT1, a novel finding that suggests the possibility that ACEI impact on the absorption of other drugs and amino acids.

### Intestinal ACE2 and its role as accessory protein for luminal amino acid transporters

ACE2 has multiple roles in the human body [47]. It is an enzyme that is expressed in various cells of many organs and is key to the regulation of local and systemic angiotensin II levels. Next to its role within local and systemic renin angiotensin systems, ACE2 has also the function of an intestinal brush-border peptidase involved in protein digestion, similarly to ACE. Indeed, high ACE2 expression levels are found at the luminal surface of small intestinal enterocytes [5] and also of kidney proximal tubule cells [53]. In small intestine, its role at the brush-border membrane is that of cleaving single carboxy-terminal AAs from nutrient proteins/peptides and also of interacting within digestive complexes comprising AA transporters, such as $B^0AT1$ [74]. Whereas intestinal $B^0AT1$ surface expression depends on the presence of ACE2 [5], its interacting partner in the kidney, collectrin [46], does not have a peptidase domain [47]. It is possible that $B^0AT1$ evolutionary first interacted with ACE2 within a complex of proteins involved in intestinal absorption named ‘metabolon’ by Broer and co-workers [74]. If this was the case, the newer kidney structural partner collectrin could have become necessary to separate the AA transport associated function from that of degrading kidney proximal tubule angiotensin II. Similarly to $B^0AT1$, the system IMINO transporter SIT1 has been suggested to depend on intestinal ACE2 and proximal tubule collectrin expression [9,46]. Indeed, high-affinity intestinal L-proline absorption was shown to be reduced in *ACE2* knock-out mice [9], and *collectrin* knock-out mice showed reduced proximal tubule SIT1 expression and consequently prolinuria [46]. We hereby show, using the *Xenopus* oocyte expression system, a functional interaction of human SIT1 with both accessory human proteins ACE2 and collectrin, as previously demonstrated for human $B^0AT1$ [53]. A functional interaction of SIT1 with ACE2 had previously not been observed when using the mouse

orthologs in *Xenopus* oocytes, presumably because of the high transport activity induced by mouse SIT1 in the absence of an exogenous accessory protein[53]. An analogous species difference had been previously observed for the functional expression of $B^0AT1$ in *Xenopus* oocytes[72].

Digestive complexes may alter transport kinetics of AA transporters, as shown for the aminopeptidase N and $B^0AT1$. Such an effect can be explained by a change in local AA concentration, but was not observed in the case of the monocarboxypeptidase ACE2 and $B^0AT1$ [74]. Similarly, we show here that the apparent affinity of SIT1 for L-proline was not changed by co-expression of either ACE2 or collectrin. Maximal transport rate ($V_{max}$) was higher upon co-expression of these accessory proteins, probably reflecting an increase in transporter expression at the oocytes' surface. Interestingly, the apparent affinity of human SIT1 for L-proline was 5 -10 times higher than previously reported for the mouse [86], rat [48] or opossum [87] orthologues, a species-specific difference for which we have no explanation.

**Axial distribution of intestinal ACE, ACE2, amino acid and peptide transporters**

Whereas the gene expression along the human intestine has been described for some transporters [77,81,88], to date information about the axial distribution of AA transporters along the digestive tract has been derived from animal studies [48,70,79]. Moreover, the data that is available from the human gut is difficult to interpret, since tissue specimens from different axial localizations originated from different patients and thus local expression differences might reflect discrepancies between patient groups [77]. This is the first study summarizing the longitudinal gene expression of several luminal and basolateral peptide and AA transporters and of ACE and ACE2 along the human digestive tract within one single group of patients. We show that the mRNA expression level of most luminal transporters is equal along the small intestine (duodenum (parts II and III) and terminal ileum) with the exception of the cysteine and dibasic AA exchanger subunit $b^{0,+}AT$. Together with the glycoprotein rBAT, this catalytic subunit forms a heteromeric AA exchanger composed of two subunits covalently linked together [40,89]. In murine intestine, both transporter subunits were shown to be expressed at a higher level in ileum than in jejunum and duodenum [70]. Similarly, we found higher levels of the transporter subunit $b^{0,+}AT$ mRNA in the distal small intestine in humans. In contrast, mRNA levels of the heavy chain rBAT were equal all along the small intestine. Basolateral 4F2hc (CD98) and luminal rBAT are the only members of the SLC3 family of heteromeric AA transporter heavy chains that (as glycoprotein subunits) associate with different members of the SLC7 family (of catalytic subunits; also called light chains), including luminal $b^{0,+}AT$ and basolateral LAT1, LAT2, $y^+LAT1$ and $y^+LAT2$ [90,91]. In small intestine enterocytes and proximal tubule kidney cells, the heterodimer $b^{0,+}AT$-rBAT mediates the apical entry of L-cystine [92] and cationic AAs including L-arginine (Arg). Here we show that the basolateral antiporter $y^+LAT1$ that exchanges cationic AAs against neutral AAs and $Na^+$ is highly expressed all along the human small intestine up to the ileum and thus most likely represents the exit pathway for transcellular cationic AA transport. In contrast, the

expression of the other $y^+$L-type transporter $y^+$LAT2 is very low, suggesting that this catalytic subunit is not of functional importance for the transepithelial cationic AA absorption. Furthermore, the mRNA expression of the basolateral AA transporters considered to be part of the neutral AA transport machinery (LAT2, LAT4 and TAT1) [93] appear to be expressed at a lower level in the terminal ileum than in the duodenum and presumably the jejunum, in contrast to $y^+$LAT1. This differential expression may reflect the fact that the largest load of neutral AAs is already absorbed before the ileum such that the efflux capacity of ileal enterocytes for neutral AAs is not anymore that high. In contrast, the cationic AA absorption via luminal $b^{0,+}$AT-4F2hc and basolateral y+LAT1 is highly developed in the terminal ileum, an observation for which we have no good explanation.

Luminal transporters - with the exception of the low affinity small neutral amino/imino acid transporter 1 (PAT1) [94] - showed very low or negligible transcript expression values in the large intestine. From the tested basolateral amino acid transporters, only the neutral and cationic AA exchanger $y^+$LAT2-4F2hc [95] and small neutral AA and L-glutamine (Gln) antiporter ASCT2 [37] were considerably expressed in the large intestine. Epithelial colonic cells show rapid renewal and transport large amounts of water and electrolytes. Colonocytes therefore need high amounts of energy and use AAs (especially Gln and L-glutamate (Glu)) from the circulation to supply the citric acid cycle [96,97]. Thus, it seems likely that basolateral exchangers ASCT2 and $y^+$LAT2-4F2hc provide colonocytes with Gln and other amino acids for their energy needs.

Whereas the dipeptidase ACE showed highest gene expression in the terminal ileum, mRNA levels of the monocarboxypeptidase ACE2 and of its interaction partners $B^0$AT1 and SIT1 were equal along the human small intestine. ACE2 protein co-localized with the transport proteins $B^0$AT1 and SIT1 to the brush-border membrane of small intestinal enterocytes on villi. Staining of $B^0$AT1 appeared to be stronger on top of the villi when compared to the signal in the crypts, supporting the notion that this surface protein is important for the function of mature enterocytes. This finding agrees with previous observations made in the rodent small intestine, showing a clear crypt-to-tip expression gradient along the villi for various AA transporters, including $B^0$AT1 [5,70,98].

**ACE-inhibitors increase expression of ACE2 and luminal amino acid and peptide transporters**

The regulation of SLC transporter expression in intestine has not been studied extensively. Physiological factors including developmental stage, diet, starvation, circadian rhythm and pathophysiological situations such as inflammatory bowel disease have been suggested to modulate intestinal SLC transporter mRNA expression. Regulatory mechanisms that may mediate these changes include transcriptional activation, RNA stabilization and epigenetic regulation [99-102].

In the present study we show that the administration of ACE-inhibitors increases small intestinal ACE2 gene expression. As yet, ACE2 up-regulation by ACEI has been reported only for other organs and in rodents [6,51]. Small intestinal ACE2 mRNA was not different in patients treated with ARBs when compared to controls. In contrast, gene expression of small intestinal ACE2 was increased in ACEI treated patients. Interestingly, gene expression levels of luminal AA transporters $B^0AT1$, PAT1 and of the peptide transporter PEPT1 were also increased in patients treated with ACE-inhibitors. The up-regulation of ACE2 mRNA correlated with that of $B^0AT1$, PEPT1 and PAT1 gene expression, indicating that these four gene products are regulated in parallel. Comparative promoter analysis detected two common promoter modules, which however appear not to suffice for mediating the observed co-regulation (data not shown). This ACEI-induced regulation did not impact on steady state blood plasma and urine AA concentrations (data not shown).

This study shows the brush-border membrane co-localization of the carboxypeptidase ACE2 and the amino acid transporters $B^0AT1$ and SIT1, as well as the axial distribution of ACE, ACE2 and most known amino acid and peptide transporters expressed in the small intestine. The gene expression levels of $B^0AT1$, PAT1 and PEPT1 are additionally shown for the first time to be regulated by treatment with ACE-inhibitors. Since the AA transporter PAT1 and the peptide transporter PEPT1 are known to transport different drugs including Vigabatrin, 5-aminolevulinic acid (PAT1) [94], Betalactam antibiotics and Valacyclovir (PEPT1) [103], we may speculate that treatment with ACE-inhibitors might impact on the absorption kinetics of these medications. Additionally, since *ACE2*-deficient mice lacking brush-border membrane $B^0AT1$ expression were shown to be more susceptible to intestinal inflammation because of impaired local L-tryptophan (Trp) homeostasis [85], it also may be that treatment with ACEIs and consecutive up-regulation of intestinal ACE2 and $B^0AT1$ reduces susceptibility to intestinal inflammation, especially in conditions of low plasma Trp levels such as observed in states of malnutrition as in anorexia nervosa [104].

**Competing interests** None.

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**Ethics approval** Ethics approval was provided by the Ethics commission of the Kanton of Zurich (EK-1744)

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**Figure Legends**

**Figure 1. Functional interaction of amino acid transporter SIT1 with accessory proteins ACE2 and collectrin. A.** *Xenopus laevis* oocytes were injected with human SIT1 (white bar), human SIT1 plus human ACE2 (dark grey bar), human SIT1 plus human collectrin (black bar), human ACE2 (third last bar; light grey), or human collectrin (second last bar; light grey) cRNA. The last bar (light grey) represents non-injected oocytes. L-proline transport was determined 2 - 3 days after injection. Each bar represents the mean transport rate ± SEM. (n = total of 40 oocytes analysed in 4 independent experiments). * $p < 0.05$ (Bonferroni corrected p values for the indicated comparisons were calculated using a mixed model including the experiment number as random factor to take into account the grouping structure of the data caused by the data acquisition in 4 independent experiments). **B.** The half maximal uptake rate $K_{0.5}$ and maximal transport capacity $V_{max}$ of L-Proline by human SIT1 was assessed in the absence (SIT1; squares; dashed line) or presence of human ACE2 (SIT1 + ACE2; triangles; dotted line) or human collectrin (SIT1 + collectrin; diamonds; continuous line). Uptake rates using 3 - 4 different L-proline concentrations, ranging from 3.3 – 100 µM, were determined 2 - 3 days after injection. Each data point represents the mean transport rate ± SEM. (n = total of 24 oocytes from 4 independent experiments). $K_{0.5}$ was similar in all three groups ($K_{0.5}$ SIT1: 40.5 ± 17.8 µM, $K_{0.5}$ SIT1 + ACE2: 47.9 ± 19.2 µM, $K_{0.5}$ SIT1 + collectrin: 42.1 ± 19.3 µM). Maximal transport capacity ($V_{max}$) was about 3-fold higher in oocytes co-injected with ACE2 or collectrin, respectively ($V_{max}$ SIT1: 34.6 ± 6.1 pmol/h/oocyte, $V_{max}$ SIT1 + ACE2: 93.0 ± 15.9 pmol/h/oocyte, $V_{max}$ SIT1 + collectrin: 91.7 ± 17.3pmol/h/oocyte). The difference of the respective $V_{max}$ is significant on a significance level of 5% which can be deduced from the non-overlapping 95% confidence intervals.

**Figure 2. Immuno-localization of ACE2 with $B^0$AT1 and SIT1 in human small intestine.** Representative tissue specimens from duodenum show $B^0$AT1 (**A**; green) and ACE2 (**B**; red) co-localizing (**C**; yellow) at the brush-border membrane of enterocytes lining small intestinal villi. The imino transporter SIT1 (**D**; green) and ACE2 (**E**; red) co-localize (**F**; yellow) at the apical membrane of epithelial cells lining duodenal villi. Cellular DNA (**A, B, D, E;** DAPI) is shown in blue to display the nuclei. Pictures were taken at 20x magnification. The white bars represent 100µm (**A, B, C**) or 20 µm (**D, E, F**), respectively. V = villi. C = crypts.

**Figure 3. Relative mRNA abundance of AA- and peptide transporters, ACE and ACE2 along the human intestine. A.** Apical (facing the lumen of the gut) AA- and peptide transporters, ACE and ACE2. **B.** Basolateral (facing the extracellular space) AA transporters. Each bar indicates the mean relative mRNA expression ± SEM (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)). mRNA expression of each gene is shown at four different intestinal localizations: duodenum, part II (white bars), part III (light grey bars), terminal ileum (dark grey bars) and ascending colon (black bars).

**Figure 4. A. Effect of ACEIs and ARBs on human duodenal ACE2 and transporter gene expression.** Mean duodenal mRNA expression (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)) of ACE2, AA transporters $B^0AT1$ and PAT1 and peptide transporter PEPT1 in control patients (control; white bars) vs. patients treated with ACE-inhibitors (ACEI; grey bars) vs. patients treated with angiotensin II $AT_1$ receptor blockers (ARB; black bars). * $p < 0.05$; ** $p < 0.01$ (Bonferroni corrected p values from a mixed mode including the experiment number as random factor to take into account the grouping structure of the data caused by the data acquisition in 4 independent experiments) . **B-D. Correlation analysis of ACE2 with $B^0AT1$, PEPT1 and PAT1 mRNA expression.** Mean duodenal gene expression of ACE2 with $B^0AT1$, PEPT1 and PAT1, respectively was correlated. ACE2 & $B^0AT1$: correlation coefficient 0.83, confidence interval 0.70 – 0.90, $p = 1.38 \times 10^{-11}$ (Figure 4B). ACE2 & PEPT1: correlation coefficient 0.80, confidence interval 0.63 – 0.89, $p = 8.56 \times 10^{-9}$ (Figure 4C). ACE2 & PAT1: correlation coefficient 0.34, confidence interval 0.02 – 0.60, p = 0.037 (Figure 4D).

**Supplementary Figure 1. Gene expression of intestinal genes with common promotor modules to ACE2, $B^0AT1$ and PEPT1.** Mean duodenal mRNA expression (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)) of HUS1, GALC, HIP1 and CDV3 in control patients (control; white bars) vs. patients treated with ACE-inhibitors (ACEI; black bars). * $p < 0.05$ (student's unpaired two-tailed *t*-test).

**Supplementary Figure 2. Blood plasma and urine amino acid levels in treated and control patients.** Blood plasma (**A**) and urine (**B**) amino acid levels from control patients (white bars) vs. patients treated with ACE-inhibitors (ACEI; grey bars) vs. patients treated with angiotensin II $AT_1$ receptor blockers (ARB; black bars). AA concentrations in the urine are given relative to creatinine. No significant differences between groups were detected. (ANOVA with post-hoc Dunnett's multiple comparison test).

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**TABLES AND FIGURES**

**Table 1. Patients data.**

| | Control[1] | ACEI[2] | ARB[3] | ACEI vs. Control | ARB vs. Control |
|---|---|---|---|---|---|
| Age [years] | 51.5 ± 2.7 | 64.4 ± 3.2 | 65.2 ± 2.0 | ** | ** |
| Gender (m/f) | 13/11 | 4/5 | 4/9 | | |
| BMI[4] [kg/m$^2$] | 25.2 ± 0.75 | 26.4 ± 1.9 | 26.5 ± 1.5 | n. s. | n. s. |
| MAP[5] [mmHg] | 87.9 ± 2.5 | 87.1 ± 3.3 | 90.3 ± 3.9 | n. s. | n. s. |
| ACE activity [U/l] | 35.2 ± 3.4 | 14.8 ± 1.7 | 33.0 ± 4.4 | ** | n. s. |
| Renin [mU/l] | 12.2 ± 1.8 | 33.1 ± 9.1 | 613 ± 392 | n. s. | n. s. |
| Aldosterone [ng/l] | 102 ± 14.2 | 69.6 ±9.5 | 148 ± 32.9 | n. s. | n. s. |
| Angiotensin I [ng/ml] | 0.49 ± 0.083 | 0.43 ± 0.077 | 0.59 ± 0.17 | n. s. | n. s. |
| Angiotensin II [pg/ml] | 11.3 ± 3.4 | 9.8 ± 3.7 | 65.0 ± 40.1 | n. s. | n. s. |

[1]patients without RAS-active medication, [2]patients treated with ACE-inhibitors, [3]patients treated with angiotensin II $AT_1$ receptor blockers, [4]BMI = body mass index, [5]MAP = mean arterial pressure. ** $p < 0.01$; n. s. = not significant ($p > 0.05$) (ANOVA with posthoc Dunnett's test)

## Supplementary Table 1. Primers and Probes used for Real-time PCR.

| Name | Start | Forward Primer | Stop | Start | Reverse Primer | Stop | Start | Probe | Stop |
|---|---|---|---|---|---|---|---|---|---|
| **Apical AA- and peptide transporters, ACE and ACE2** | | | | | | | | | |
| **$B^0AT1$ (SLC6A19)** | 1646 | GTG TGG ACA GGT TCA ATA AGG ACA T | 1670 | 1719 | CCA CGT GAC TTG CCA GAA GAT | 1699 | 1676 | TCA TGA TCG GCC ACA AGC CCA A | 1697 |
| **SIT1 (SLC6A20)** | 5134 | GCA TTC CCT CCA CGA TAA GC | 5153 | 5227 | GGC AAC GTG TGG CGA TTC | 5210 | 5169 | ACC AGG AAA CTA CCT CCG CCG TAC ATC T | 5196 |
| **PAT1 (SLC36A1)** | 1057 | GTC CCG GCT GAG ATC ATC A | 1075 | 1173 | GAT GGC CAA GAT GCA TGT CA | 1154 | 1090 | TCC CGA GCG CCC GAG CAC | 1107 |
| **$b^{0,+}AT$ (SLC7A9)** | 630 | TCC TCA AAT CGT TGT GAA ATG C | 651 | 707 | AGC CGC ACG CTC AGT GA | 691 | 662 | CCG CCA TCT TGT TCA TCT CGA CAG TGA | 688 |
| **rBAT (SLC3A1)** | 1014 | CAC GGT CAC ACA ATA CTC GGA | 1034 | 1153 | CTT CAG TCC CCA TGA ACC TGT | 1133 | 1036 | CTG TAC CAT GAC TTC ACC ACC ACG CA | 1061 |
| **PEPT1 (SLC15A1)** | 335 | TGG ACA AGC AGT CAC CTC AGT AA | 357 | 427 | AGC ACC ACG TGC ACA GGA A | 409 | 382 | ACA ACC ATG ATG GCA CCC CCG A | 403 |
| **ACE** | 625 | ACC GCT GTA CGA GGA TTT CAC T | 646 | 764 | CTA GCT GTT GGT AGA GGT GTT CCA | 741 | 665 | TAC AAG CAG GAC GGC TTC ACA GAC ACG | 691 |
| **ACE2** | 1536 | GGG AGA TGA AGC GAG AGA TAG TTG | 1559 | 1636 | TGA GTA ATC ATT AGA AAC ATG GAA CAG A | 1609 | 1580 | CAT GAT GAA ACA TAC TGT GAC CCC GCA | 1606 |
| **Basolateral AA transporters** | | | | | | | | | |
| **LAT1 (SLC7A5)** | 758 | CCT ATG GAG GAT GGA ATT ACT TGA A | 782 | 906 | GGT GGA CAG GGT GGT GAA GTA G | 885 | 784 | TTC GTC ACA GAG GAA ATG ATC AAC CCC T | 811 |
| **LAT2 (SLC7A8)** | 952 | ATG CCC ATT TCT GTT GCC C | 970 | 1084 | AGC GCT TCA CGT GGA TCA T | 1066 | 987 | AGT TAA TGG GTC TCT CTT CAC CTC CTC TCG G | 1017 |
| **LAT4 (SLC43A2)** | 1537 | GAC CCT CTG TGG GTG AAC GT | 1556 | 1677 | GAT TTT GAG GAA GAG TTT GTC ATC CT | 1652 | 1579 | CTG GGC TTC TGC CTC CCG CTC | 1599 |
| **$y^+LAT1$ (SLC7A7)** | 1823 | CCC TCA AGC TCA GCG TTT T | 1842 | 2316 | TAA AGT GGA ACA GCC ACC AGG | 2337 | 1847 | CCG ATT GTC TTC TGC CTC TGC ACC AT | 1873 |
| **$y^+LAT2$ (SLC7A6)** | 1222 | CAG CTC TAC CTC CGC TGG AA | 1241 | 1312 | ACA CGG AGC ATA TGC AGA ACA C | 1291 | 1256 | CCC GGC CTC TCA AGC TGA GCG | 1276 |
| **4F2hc (SCL3A2)** | 708 | CAG AAG GAT GAT GTC GCT CAG A | 729 | 813 | GGA TGC TCT TTT TTT TAG CCG ATT | 790 | 734 | CTT GCT GCA GAT CGA CCC CAA TTT TG | 759 |
| **ASCT2 (SLC1A5)** | 532 | AAG GAG GTG CTC GAT TCG TTC | 552 | 615 | AGA GTA TGA GCG AAA GGC TGC | 595 | 563 | CGA GAA ATA TCT TCC CTT CCA ACC TGG TGT CA | 594 |
| **TAT1 (SLC16A10)** | 278 | TGC TCT TCG TGT CCA TGC T | 296 | 404 | AAG ACG CTG ACT ATT GGG CAG | 384 | 299 | AAA CCT TCG GCT CCA AAG ACG ATG ACA | 325 |
| **Housekeeping Genes** | | | | | | | | | |
| **Villin** | 2002 | AGG ATG ATG TGT TCC TAC TAG ATG TCT G | 2029 | 2092 | GTT TCT GCG GCC TTC TTC | 2075 | 2031 | TGT TTC CCA ATC CAG AAG AAG ACC TGG TC | 2059 |
| **HPRT** | 210 | TTA TGG ACA GGA CTG AAC GTC TTG | 233 | 323 | CCA GCA GGT CAG CAA AGA ATT | 303 | 262 | CCATCACATTGTAGCC CTCTGTGTGCTC | 289 |
| **Intestinal Genes with common promoter modules to ACE2, $B^0AT1$ and PEPT1** | | | | | | | | | |
| **HUS1** | 371 | CCG TGG AGC TGT TAT CTA TGT CAA | 394 | 475 | CCA CCG GTT CTT GTA AGT CCT T | 454 | 403 | CGC ATT GTG ACC CAT GAC ATC CCC | 426 |
| **GALC** | 2065 | CAC CAC TCG TAT CCT CGG AAA | 2085 | 2188 | TTG GTG CCT CTT TGC ATA TTT TAA | 2165 | 2126 | TGC CGT CTG TTG TCT GCC CAT CAC | 2149 |
| **HIP1** | 111 | GCG GAC TCA GAC TGT CAG CAT | 131 | 250 | GCA GGC GGT TGA CAA CAG A | 232 | 180 | CAG AAC GTG CAT ACT GGG CAC CCA | 203 |
| **CDV3** | 546 | ACC ACA AGG ACC ACC AGA AAT C | 567 | 633 | TTT ATC CTT CCG GCT TTC TAC ATG | 610 | 573 | TGA TAC ACA GTT CCC ATC CCT GCA GTC A | 600 |

**Figure 1**

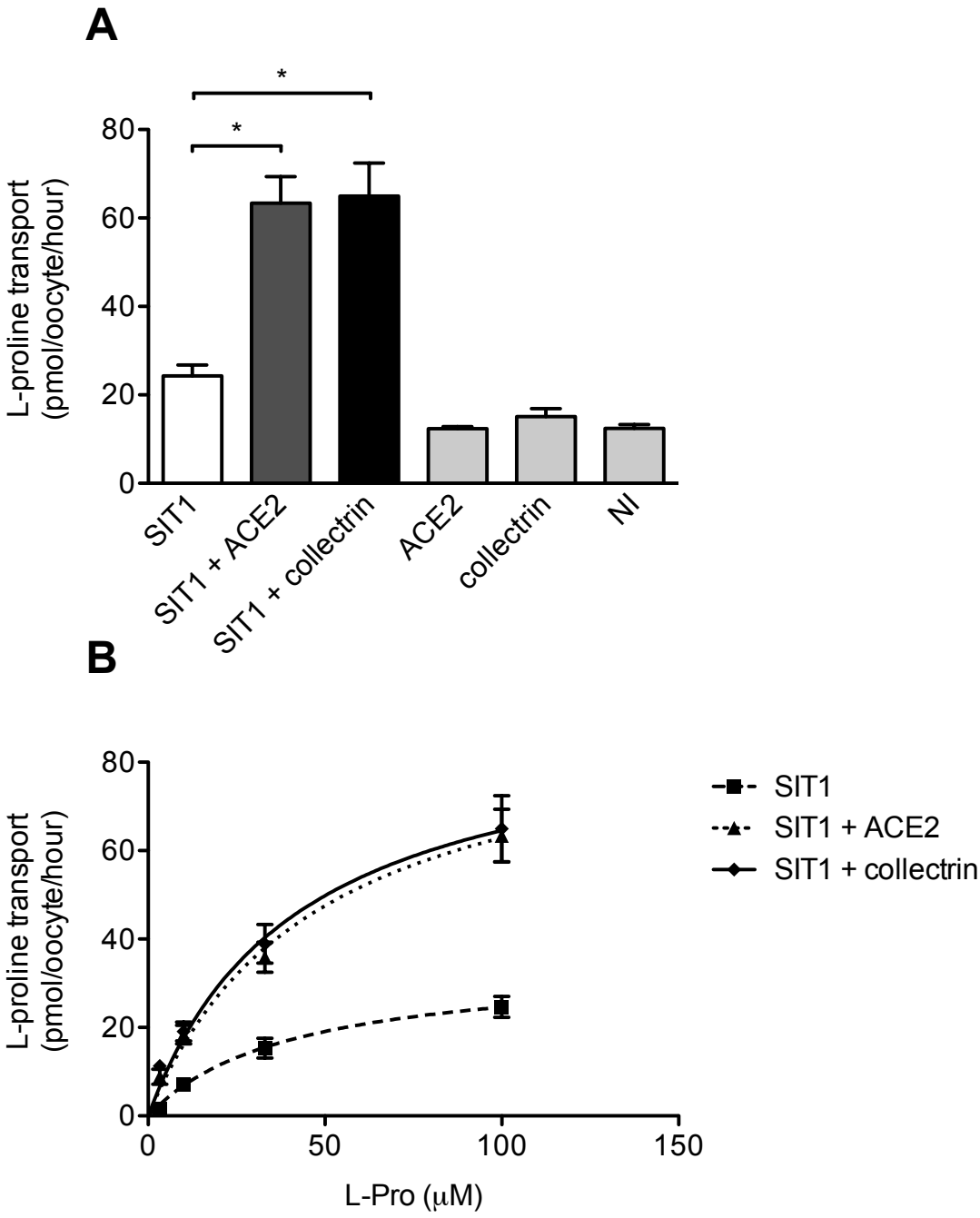

A
*
*
80
60
40
20
0
L-proline transport
(pmol/oocyte/hour)
SIT1
SIT1 + ACE2
SIT1 + collectrin
ACE2
collectrin
NI
B
80
60
40
20
0
0
50
100
150
L-Pro (μM)
L-proline transport
(pmol/oocyte/hour)
SIT1
SIT1 + ACE2
SIT1 + collectrin

**Figure 2**

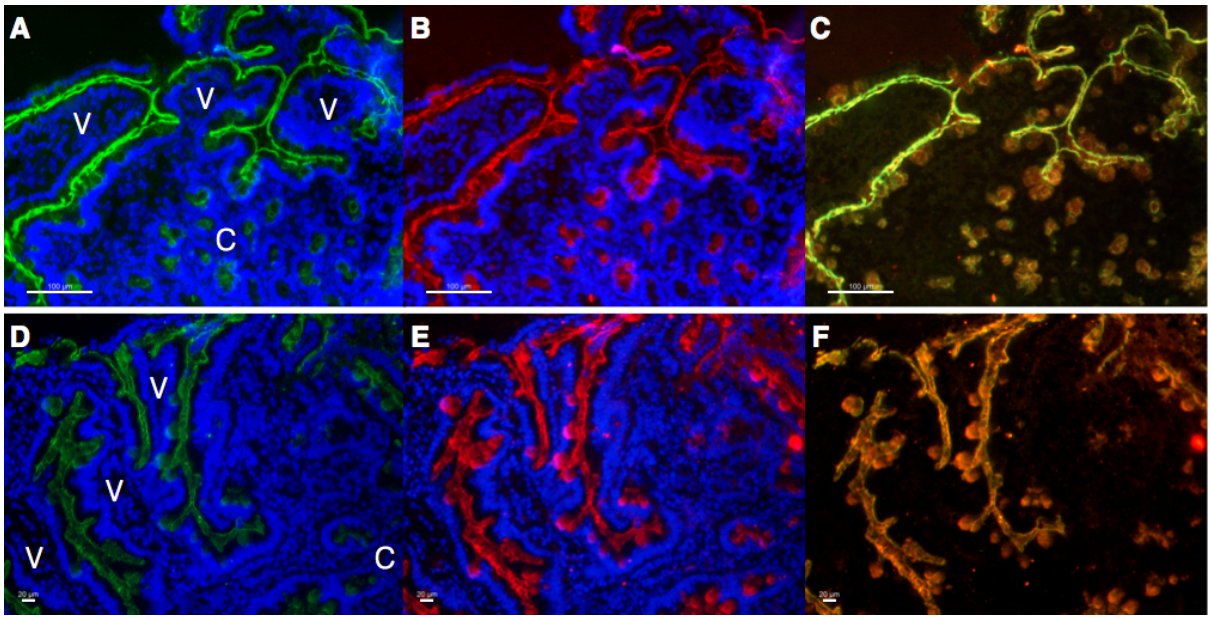


**Figure 3**

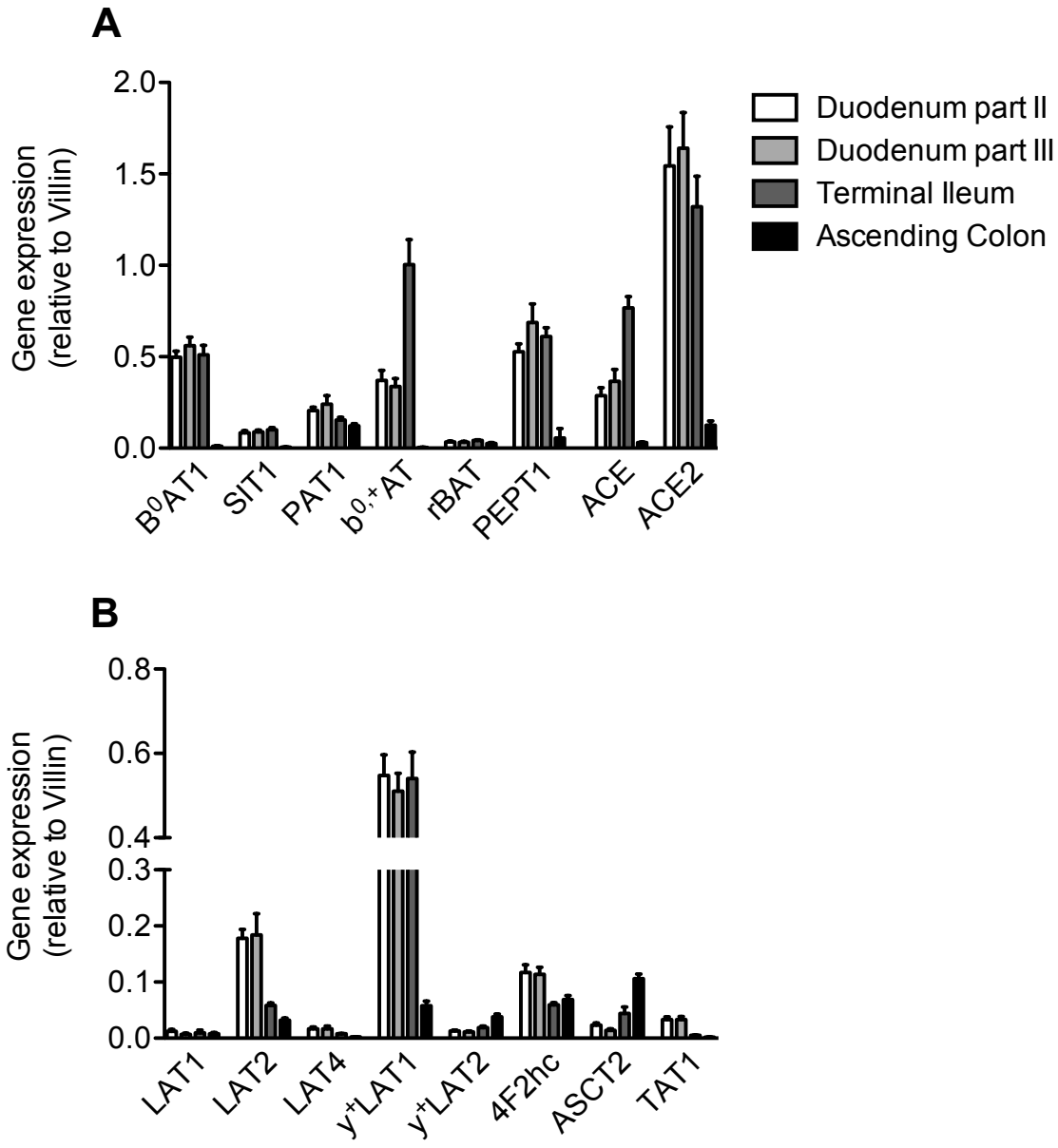
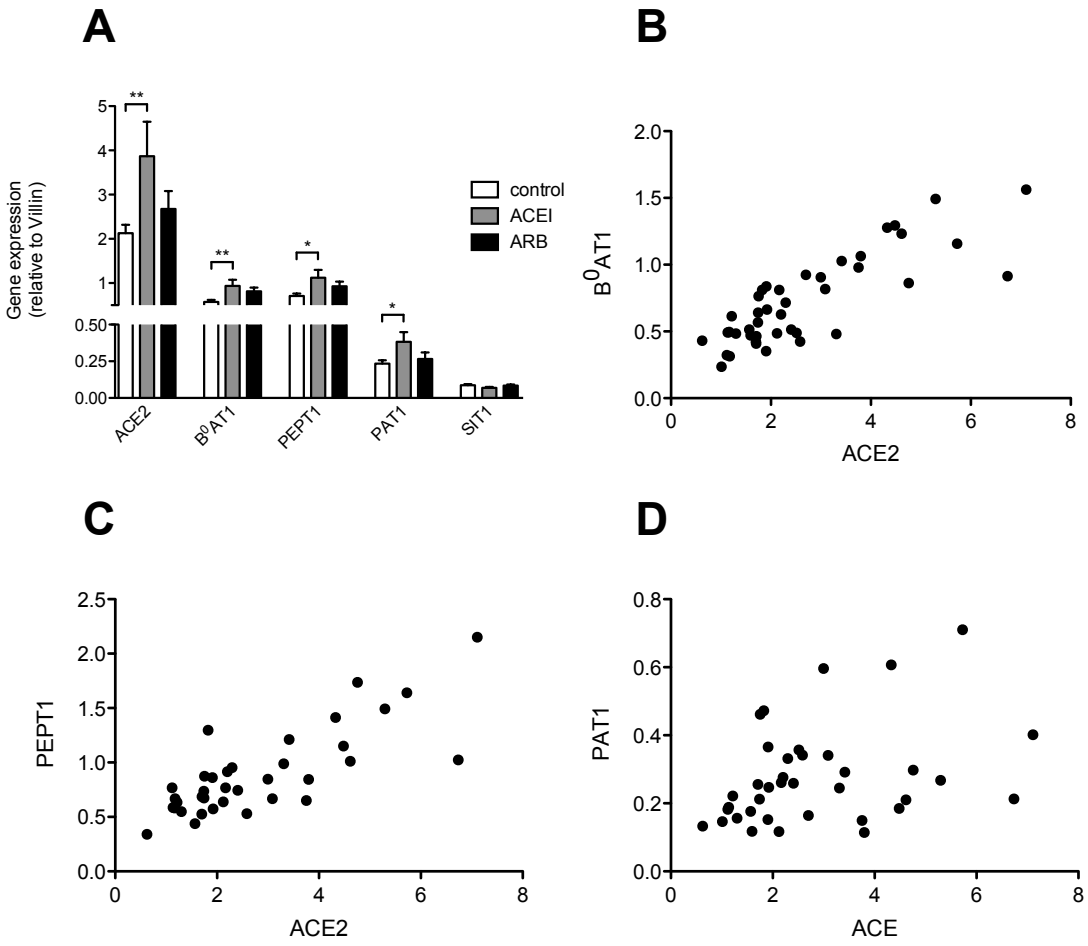
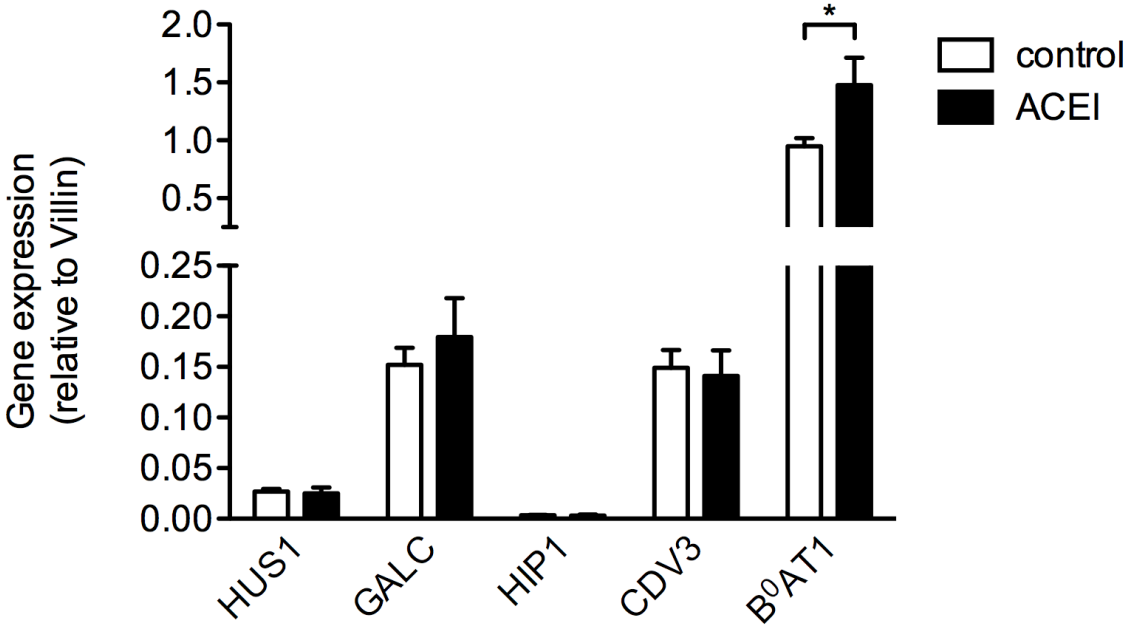

A
Gene expression (relative to Villin)
2.0
1.5
1.0
0.5
0.0
B0AT1
SIT1
PAT1
b0,+AT
rBAT
PEPT1
ACE
ACE2
Duodenum part II
Duodenum part III
Terminal Ileum
Ascending Colon
B
Gene expression (relative to Villin)
0.8
0.6
0.4
0.3
0.2
0.1
0.0
LAT1
LAT2
LAT4
y+LAT1
y+LAT2
4F2hc
ASCT2
TAT1

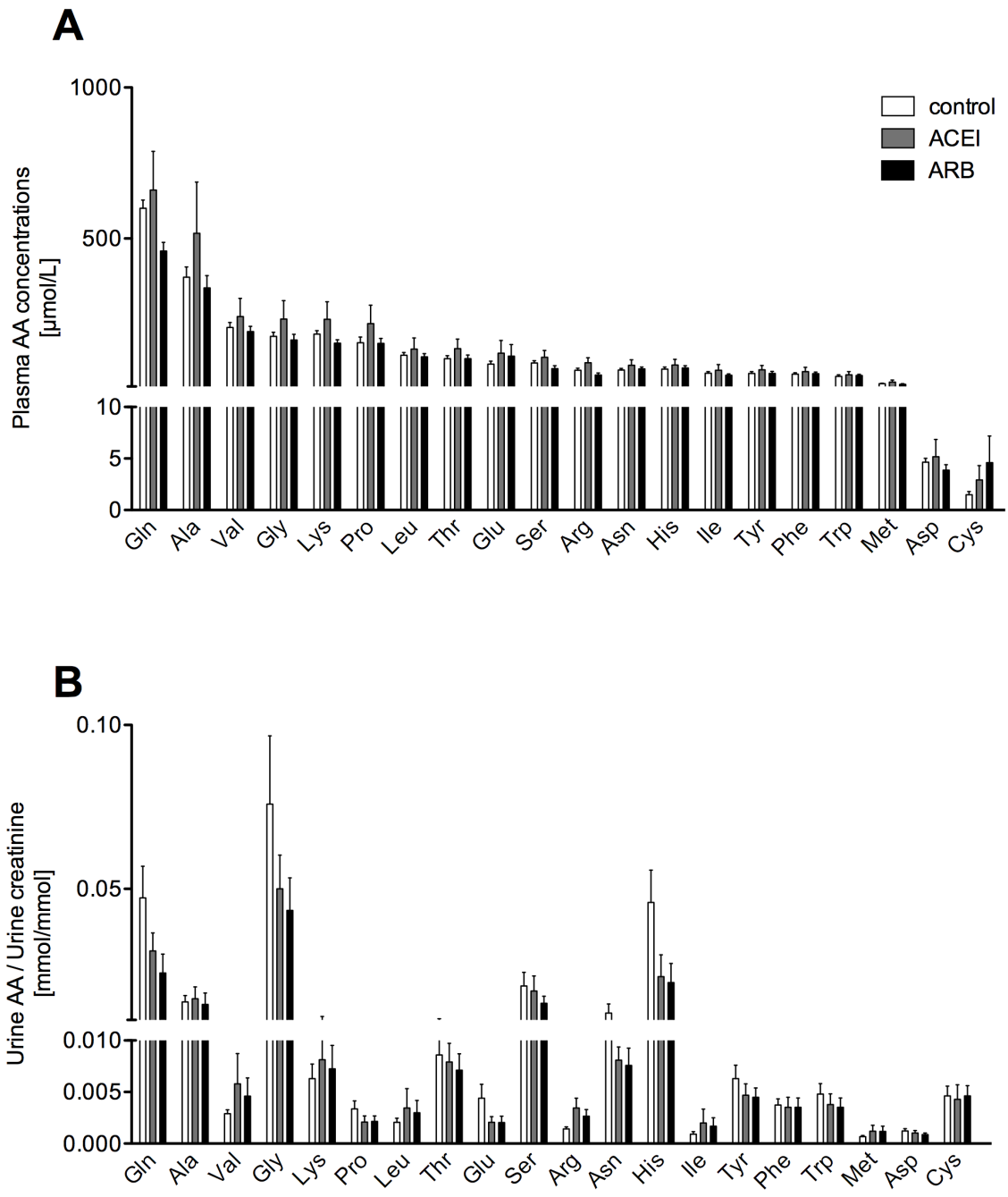
**Figure 4**


A
B
C
D
Gene expression (relative to Villin)
control
ACEI
ARB
ACE2
B0AT1
PEPT1
PAT1
SIT1
B0AT1
ACE2
PEPT1
ACE2
PAT1
ACE

**Supplementary Figure 1**



**Supplementary Figure 2**



# 6. Manuscript: *'Intestinal monosaccharide transporter expression in diabetic patients – effect of metformin treatment'*

This section contains the manuscript *'Intestinal monosaccharide transporter expression in diabetic patients – effect of metformin treatment'.*

When the present thesis was written, some experiments including the subcellular localization of GLUT2 by immunofluorescence and the gene expression of hepatocyte nuclear factors 1α, 4α and 3β (HNF-1α, HNF-4α and HNF-3β) were still pending.

Title (160 characters)

**Intestinal monosaccharide transporter expression in diabetic patients – effect of metformin treatment**

Authors

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*Annotation: The reference numbers within this manuscript refer to the references listed in chapter 10.*

**Abstract**

Type 2 diabetes mellitus (T2DM) is a common disease of glucose metabolism characterized by hyperglycemia that may cause severe complications. Metformin is the most widely used and first-line pharmacotherapy for T2DM. Dietary carbohydrates are absorbed in the small intestine as monosaccharides across the epithelial enterocytes, crossing sequentially the luminal membrane via either the sodium transporter SGLT1 (SLC5A1) or the facilitated diffusion protein GLUT5 (SLC2A5) and the basolateral membrane presumably via GLUT2 (SLC2A2). Both experimental diabetes mellitus and metformin treatment were shown to affect small intestinal monosaccharide transporter expression in rodents. Furthermore one study performed in humans indicated that impaired fasting glucose correlated with changed mucosal small intestinal hexose transporter expression. To investigate in human small intestine the potential impact of type 2 diabetes mellitus with and without additional metformin treatment on hexose transporter expression, intestinal biopsies taken by routine endoscopy from 46 patients, whereof 10 where diabetics under metformin treatment and 5 were non-treated type 2 diabetics were tested. GLUT2, GLUT5 and SGLT1 gene expression showed a proximal to distal axial decrease along the human digestive tract as assessed by real-time PCR. Immunofluorescence staining localized GLUT5 exclusively to the luminal enterocyte membrane. GLUT2 mRNA levels were almost 2-fold higher in human small intestine of non-treated diabetics when compared to metformin-treated diabetics and non-diabetic controls respectively. A similar trend was not statistically significant for GLUT5. Taken together this study characterized in human intestine the axial and subcellular localization of intestinal hexose transporters and suggests that GLUT2 expression is increased in T2DM, an effect that is abolished by metformin treatment.

**Introduction**

Type 2 diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, caused by insulin resistance of fat and muscle cells, inadequate suppression of glucose production in the liver in response to insulin and by the inability of pancreatic islet β-cells to secret adequate amounts of insulin [10,105]. Its increasing prevalence and devastating effects on many organs including cardio-vascular disease, renal failure, vision loss and nerve damage [102] make it an enormous healthcare and economic burden [105,106]. According to the World Health Organization (WHO) and the American Diabetes Association (ADA) diabetes mellitus is currently defined as fasting plasma glucose levels ≥ 7.0 mmol/l (126 mg/dl) and/or plasma glucose levels ≥ 11.1 mmol/l (200 mg/dL) two hours after a 75 g oral glucose load as part of a glucose tolerance test [58]. Since 2009, glycosylated hemoglobin $A_{1c}$ ($HBA_{1c}$) concentrations higher than 6.5% have been added to these diagnostic criteria [58,59]. Currently, various international professional advisory boards and healthcare authorities adopted these recommendations [105].

Management of type 2 diabetes mellitus relies on lifestile approaches (consisting of increased physical activity, reduction of dietary carbohydrate intake and loss of body weight) and the use of hypoglycemic agents lowering blood glucose levels [107]. The biguanide metformin hereby is the most widely used and first-line pharmacotherapy for type 2 diabetes mellitus [11,14,60]. The glucose-lowering effect of metformin is mainly attributed to decreased hepatic glucose output and increased peripheral (i.e. fat and muscle tissue) glucose uptake. Other mechanisms of metformin function are reduced fatty-acid oxidation and increased intestinal use of glucose [12,13]. Furthermore, metformin treatment seems to exert an inhibitory effect on intestinal glucose absorption as shown in rats [14], but inconsistent effects of metformin on intestinal monosaccharide transporter expression were found in non-diabetic rodents [61,62].

After enzymatic digestion by the pancreatic α-amylase and brush-border membrane (BBM)-bound disaccharidases, dietary carbohydrates are absorbed exclusively as monosaccharides including D-glucose (Glu), D-galactose (Gal) and D-fructose (Frc). Three different intestinal transporters, SGLT1 (SLC5A1), GLUT2 (SLC2A2) and GLUT5 (SLC2A5) are known to mediate intestinal monosaccharide absorption [2,3]. SGLT1 refers to a high affinity, low capacity sodium- dependent BBM glucose and galactose symporter. GLUT2 and 5 are facilitated diffusion protein uniporters with GLUT2 being expressed in the basolateral enterocyte membrane, transporting Glu, Gal and Frc and GLUT5 transporting Frc across the BBM. In experimental diabetes, but also in fructose- and/or glucose rich diets in rodents [18], as well as in morbidly obese human subjects [19] the basolateral high-capacity, low-affinity facilitative glucose, fructose and galactose transporter GLUT2 is also expressed in the apical enterocyte membrane [18]. This finding is in accordance with the fact that glucose absorption in humans increased at luminal concentrations far above those required to saturate the high affinity, low capacity glucose and galactose transporter SGLT1 [18], but remains selective in

these conditions, such that it is likely transporter-mediated and not due to a paracellular leak.

Both, specific diets and (experimental) diabetes affect small intestinal monosaccharide transporter expression. Dietary fructose and sucrose increase small intestinal SGLT1, GLUT2 and GLUT5 expression in non-diabetic rats [17]. Whereas expression of the fructose transporter GLUT5 seems to be specifically responsive to fructose, intestinal expression of SGLT1 and GLUT2 also depend on dietary glucose [63]. The regulation of intestinal hexose transporter in diabetic subjects seems to be more complex and led to contradictory findings: Whereas one study in humans showed increased gene and protein expression of intestinal hexose transporters GLUT2, GLUT5 and SGLT1 in humans suffering from impaired fasting glucose levels [16], another study performed in Zucker rats – reflecting a model of type 2 diabetes – shows no effect on intestinal monosaccharide transporter GLUT2, GLUT5 and SGLT1 expression [20]. By contrast, surveys in rodents with streptozotocin-induced type 1 diabetes showed increased small intestinal GLUT2 and SGLT1 expression [64,65] with inconsistent findings concerning small intestinal GLUT5 expression [63,65].
The present study aimed at investigating the potential impact of type 2 diabetes mellitus on small intestinal monosaccharide transporter GLUT2, GLUT5 and SGLT1 expression with respect to metformin treatment. Additionally, the differential gene expression of these transporters along the human digestive tract was analyzed, *as well as the subcellular localization of GLUT2 in fasting human subjects suffering from type 2 diabetes mellitus*. (Remark: Results of GLUT2 localization were pending when the present thesis was written)

**Materials and Methods**

Study population

All patients included in the present study were examined at one single institute as a part of a routine medical checkup. Selected patients underwent gastroduodenoscopy only (n= 34), ileocolonoscopy only (n= 2) or a combined gastroduodeno-/ileocolonoscopy (n= 10) in the morning after overnight fasting in order to avoid short-time dietary- or day-time effects on intestinal monosaccharide gene expression. During endoscopy, 8 mucosal biopsies were obtained at 4 different intestinal localizations, namely from the descending (part II) and inferior (part III) duodenum as well as from the terminal ileum and from the ascending colon. In total, 46 patients (26 female, 20male) were included the current study. 12 patients had a positive history for diabetes mellitus type II and 3 patients exhibited HbA1c levels ≥ 6.5%. 10 of 15 diabetic patients were under metformin treatment. 31 patients with negative history for type 2 diabetes mellitus, HbA1c levels < 6.5% and spontaneous blood glucose levels < 11.1 mmol/l served as non-diabetic controls. Patient's treatment was independent from the present study or hereby obtained results. Following endoscopy, blood samples were drawn from the patient's cubital vein in order to assess laboratory values of glucose metabolism (spontaneous plasma blood glucose and $HbA_{1c}$ levels), and kidney function (plasmatic uric acid- and –creatinine levels as well as the calculated GFR). Physiologic parameters, including body mass index (BMI) and mean arterial blood pressure were further assessed. Included patient's age ranged from 18 to 80 years and patient's Body Mass Index (BMI) was 18 to 35 $kg/m^2$. Patients with severe pathologies of the GI- tract, such as coeliac disease, Crohn's disease and ulcerative colitis, as well as patients with carcinomas, kidney- or hepatic insufficiency, bleeding disorders, infectious diseases, oral anticoagulation, drug- or alcohol abuse, mental disability or type 1 diabetes mellitus, were excluded from the present study (Table 1).

Intestinal biopsies

After removal, tissue specimens were immediately (within 10 seconds) frozen in liquid nitrogen and stored at $-80^0$C until further processing.

RNA extraction

RNA was extracted by disrupting the tissue with MagNA Lyser Green Beads (from Roche, Switzerland) for 30 seconds at 6000 rpm (utilizing a Precellys® 24 tissue homogenizer from Bertin Technologies, France) in 350 µl RLT-Beta-Mercapto-Ethanol buffer (10µl β-ME/1 ml RLT-buffer). The obtained dilution was then centrifuged at 10°C for 5 minutes at 10000g. RNA was isolated from the supernatant using the Qiagen RNeasy mini kit (from QIAGEN, Switzerland).

RNA concentrations at 260nm wavelength and purity of the extractions were measured with spectrophotometry applying the NanoDrop 1000 Spectrophotometer (from Witec AG,

Switzerland).

Reverse transcription

Reverse transcription was performed using the Biometra T Gradient Thermocycler (from Biolabo Scientific Instruments SA, Switzerland) and Applied Biosystems Taq Man RT-PCR reagents and at final concentrations in the reaction mix of: Random hexamers (2.5µM), $MgCl_2$ (5mM), RT buffer (1x), deoxyNTP mix (500 µM each), RNAse inhibitor (0.4 U $µl^{-1}$), multiscribe reverse trancriptase enzyme 1.25 U $µl^{-1}$), RNA template (33 ng $µl^{-1}$) and RNAse free water. In parallel to all reactions negative control experiments were performed by omitting the multiscribe reverse trancriptase enzyme. Finally, samples were stored at -20°C until further processing.

Primers and probes

Primers and probes were designed using the software Primer Express 3.0 (from Applied Biosystems, Switzerland) or according to a previous report[80]. Self-designed primers bridged intron-exon boundaries to avoid a contamination by genomic DNA and amplified fragments base pair length was 70 to 100. PCR of all used primers was performed and resulted in a single product of expected base pair length using (data not shown). Probes were labelled at the 5' end with the fluorescent reporter dye FAM and at the 3' end with the quencher dye TAMRA (from Microsynth AG, Switzerland) (Table 2).

Real time PCR

Quantitative Real Time PCR (qRT-PCR) was perfomed by preparing a 20µl PCR reaction volume containing TaqMan Universal PCR Master Mix (10µl) (from Applied Biosystems AG, Switzerland), Primers (0.8µl each; final concentration: 1µM each), Probe (0.4µl; final concentration: 0.1µM) cDNA (1µl), and DEPC-Water (7µl). 96-well optical reaction plates and the 7500 Fast Real-Time System Termocycler (from Applied Biosystmes AG, Switzerland) were utilized. In total 45 thermal cycles were performed (set at 95°C for 10 minutes, 60°C for 15 seconds and 72°C for 1 minute). Individual thresholds were chosen for each gene within in the linear range of the amplicon curves. All reactions were operated three times in parallel and if the maximal difference between obtained results was less than one cycle mean values were calculated. Furthermore, negative controls were run in parallel for each gene and obtained results were only used, if the difference between the reaction (mean value of three measurements) and negative controls was more than 5 cycles. The gene expression of targed mRNA was calculated relative to villin mRNA (relative expression = $2^{-\Delta Ct}$, $\Delta Ct$ = average Ct value of target – average Ct value of villin), which was used as housekeeping gene[81]. HPRT (Hypoxanthine phosphoribosyltransferase) was used as a secondary housekeeping gene to verify the exspected consistency of villin mRNA expression along the human digestive tract (data not shown)[82].

**Immunofluorescence**

Cryosection

Intestinal biopsies were embedded in optical coherence tomography cryostat medium (from Medite Medizinaltechnik AG, Switzerland) that was put into tubes containing liquid propane. Tubes were then transferred into liquid nitrogen and blocks were cut or stored at -80°C. Slices of 5 µm thickness were cut using a cryotome (from Leica CM 1850 Cryostat, Switzerland) and transferred onto polylysine slides (from O. Kindler & CO GmBH, Germany). Slides were kept at -20°C until immunostaining.

Immunostaining

After defrosting the samples at room temperature for 5 minutes in a wet chamber, slides were immersed in 70% Methanol for 90 seconds for fixation. Thereafter 3 washing steps (1 x 15min, 2 x 5min) with phosphate buffered saline (PBS, 0.1M) (137.0 mM NaCl, 2.7 mM KCl, 12.0 mM $HPO_4^{2-}/H_2PO_4^-$) were performed. The tissue was then kept in 2% bovine serum albumin (BSA) diluted in PBS (0.1M) for 1 hour at room temperature in order to reduce unspecific antibody binding. Subsequently, primary antibodies were added to the samples for another 1 hour at room temperature. Hereby, the following two antibodies were used: 1. Affinity purified rabbit anti human polyclonal GLUT2 (from Abcam, United Kingdom)) and 2. Affinity purified rabbit anti human polyclonal GLUT5 (Alpha Diagnostic, San Antonio, Texas). Primary antibodies were diluted 1:100 in PBS (0.1M) enriched with 2% BSA and 0.04% Triton X-100. Then, the sections were washed again with PBS (0.1M) (3 x 5min) and subsequently, the secondary antibody (Alexa Fluor® 488 donkey anti-rabbit IgG, dilution 1:500 and 4', 6'-diamidino-2-phenyl- indole (DAPI, Merck, NJ) (0.1mg/ml) (diluted 1:5'000) was added for 1 hour at room temperature. Sections were rinsed again in PBS (0.1M) (3 x 5min) before mounting with DAKO fluorescence mounting media (Dako Cytomation, Baar, Switzerland). The tissue specimens were finally examined utilizing a Nikon Eclipse TE300/200 inverted microscope fitted with a DS-5M Standard charge-coupled device camera. Pictures were captured using the NIS-Elements software (from Nikon Instruments Inc., Melville, New York) and processed with the Adobe Photoshop CS5 software. In parallel – as a negative control – sections were processed the same way without adding a primary antibody not yielding a detectable signal (data not shown).

Statistical analysis

For data representation, descriptive statistics and statistical analysis, the statistical software Graphpad Prism 5 was used. Data are shown as mean ± SEM (standard error of the mean). For multiple comparisons, an ANOVA (one-way analysis of variance) with post-hoc Bonferroni's Multiple Comparison test was used. *p*-values ≤ 0.05 were considered statistically

significant.

Ethics

The present study was performed according to Good clinical practice guidelines and was approved by the local ethics committee. Patients were informed at least 24 hours before endoscopy about the present study and written informed consent was obtained from each patient.

**Results**

Patients characteristics

In total, 46 patients (10 patients suffering from type 2 diabetes mellitus with metformin treatment, 5 non-treated diabetics and 31 non-diabetic controls) were included in the present study. 12 of 15 diabetic patients had a positive history of diabetes mellitus type 2 and 3 of 15 patients were rated as 'diabetic' based on $HbA_{1c}$ values of 6.5 or higher. Blood glucose levels were significantly higher in metformin-treated diabetics when compared to non-diabetic controls and to diabetics without metformin treatment. $HbA_{1c}$ levels were significantly higher in metformin-treated type 2 diabetic patients when compared to non-diabetic controls. Plasma urea levels were higher in diabetics without metformin treatment when compared to non-diabetic control patients. Other parameters assessed, including body mass index, patient's age and blood creatinine levels were not different between groups (Table 1).

Monosaccharide transporter gene expression along the human digestive tract

Relative gene expression of monosaccharide transporters GLUT2, GLUT5 and SGLT1 to the housekeeping gene villin was assessed at four different localizations of the human intestine (duodenum parts II and III, terminal ileum and ascending colon) using mucosal biopsies from 10 patients referred for combined gastroduodenoscopy and ileocolonoscopy. Gene expression of facilitated diffusion protein GLUT2 was slightly, but non-significantly lower in the terminal ileum, when compared to duodenum parts II and III. Its expression in the ascending colon was below the detection limit in all 10 patients assessed. Similarly, but more pronounced, gene expression of GLUT5 was similar in duodenum parts II and III, and non-sognificantly lower in the terminal ileum. A very low GLUT5 mRNA level was seen in only 1 of 10 patients, whereas in 9 of 10 patients the GLUT5 gene could not be detected in the ascending colon. Sodium-dependent glucose and galactose transporter SGLT1 mRNA was significantly higher in duodenum parts II and III when compared to the terminal ileum and the ascending colon ($p < 0.0001$ for all four comparisons). Furthermore, gene expression of SGLT1 in the ascending colon was above the detection limit in most patients assessed and showed significantly lower expression levels than in the terminal ileum ($p < 0.01$) (Figure 1).

Duodenal monosaccharide transporter expression in metformin-treated type 2 diabetics vs. type 2 diabetics without metformin treatment vs. non-diabetic controls

Mucosal biopsies from 44 patients referred for gastroduodenoscopy were analyzed. Fifteen of these patients were diabetic and 10 of these 15 patients were under metformin treatment. Since mRNA expression levels of all 3 monosaccharide transporters (GLUT2, GLUT5 and SGLT1) assessed were not different between duodenum parts II and III (as assessed by a paired two-tailed *t*-test for each gene; data not shown), the impact of type 2 diabetes mellitus and of metformin treatment was measured in duodenal samples (means of all available biopsies) only.

The mean duodenal gene expression level of GLUT2 in non-metformin-treated type 2 diabetics was increased 1.9- and 1.7-fold, when compared to metformin-treated diabetics and non-diabetic controls, respectively (Figure 3a). Similarly, non-metformin treated patients showed a trend to increased duodenal GLUT5 mRNA levels when compared to metformin-treated diabetics and non-diabetic controls, respectively, without yielding significant differences (Figure 3b). In contrast, SGLT1 showed equal duodenal gene expression levels among groups (Figure 3c).

**Discussion**

Type 2 diabetes mellitus is a common disease of glucose metabolism that may result in severe morbidity [10]. The dimethylbiguanide metformin is the first-line antihyperglycemic medication in T2DM treatment [11]. Metfromin increases insuline-stimulated glucose uptake in muscle cells and decreases hepatic Glc output [12,13]. Furthermore, metformin treatment has an inhibitory effect on intestinal glucose absorption as shown in rodents [14]. Three different small intestinal hexose transporters, SGLT1, GLUT2 and GLUT5 provide intestinal monosaccharide absorption. Whereas SGLT1 and GLUT5 are expressed exclusively in the brush-border membrane, GLUT2 localizes to the basolateral enterocyte membrane, but shows luminal expression in conditions of experimental diabetes and in fructose- or glucose rich diets in rodents [18], as well as in in morbidly obese human subjects [19]. Whereas specific fructose and sucrose rich diets affect small intestinal SGLT1, GLUT2 and GLUT5 expression [17], the changes in small intestinal hexose transporter expression as a result of diabetes mellitus seems to be more complex [16,20].

We could show in the present study an increase in small intestinal GLUT2 mRNA expression in non-metformin treated human type 2 diabetics, an effect that was abolished uppon metformin treatment. Whereas SGLT1 expression was equal among groups (i.e. metformin-treated type 2 diabetics, non metformin-treated type 2 diabetics and non-diabetic controls), also GLUT5 gene expression was potentially increased in diabetic patients without metformin treatment (non-significant increase). We could further demonstrate for the first time the longitudinal gene expression of hexose transporters along the human digestive tract in a single cohort of patients, as well as protein localization of facilitated diffusion proteins *GLUT2* and GLUT5. Whereas the latter exclusively localized to the BBM as expected, *immunolocalisation of uniporter GLUT2 was still pending, when the present thesis was written. In experimental diabetes, as well as in fructose-/glucose rich diets in rodents, GLUT2 also showed, besides its basolateral localization, expression in the apical enterocyte membrane. We hence hypothesize that this increased translocation to the luminal enterocyte membrane might be for GLUT2, next to its raised gene expression an additional mechanism by which it contributes to fast glucose absorption in non-treated type 2 diabetics*

<u>Axial distribution of intestinal hexose transporters GLUT2, GLUT5 and SGLT1 in human subjects</u>

To the author's knowledge, this is the first study summarizing the longitudinal gene expression of intestinal hexose transporters GLUT2, GLUT5 and SGLT1 along the human digestive tract in one single cohort of patients. Unlike amino acid transporters showing variable transporter expression alongside of the human digestive tract (Vuille-dit-Bille et al, unpublisched data) all three hexose transporters exhibited at similar gene expression pattern along the human intestine: Whereas GLUT2 and GLUT5 showed a trend to decreasing

mRNA levels (without yielding statistical significance) from proximal to distal along the human small intestine with undetectable (GLUT2) or almost undetectable (GLUT5) gene expression in the large intestine, SGLT1 mRNA was significantly lower in the terminal ileum when compared to duodenum parts II or III, respectively and significantly lower in the large intestine when compared to the duodenum and the terminal ileum. This indicates that most of the monosaccharide absorption occurs already in the proximal small intestine. Concerning glucose transport along the luminal membrane - unlike in intestine - in kidney proximal tubule, the re-uptake of D-glucose from the glomerular filtrate along the brush border membrane is mainly mediated by the low affinity, high capacity transporter SGLT2 (SLC5A2) that is essentially expressed in the more proximal convoluted segments S1 and S2. High affinity, low capacity transporter SGLT1 is primarily expressed in the more distal straight segment S3 and enables the uptake of remaining luminal glucose [108]. Since in intestine only SGLT1 and GLUT2 provide transepithelial glucose transport and therefore mediate glucose absorption [109], it is not surprising, that transporter expression is highest in proximal segments, where presumably most of the glucose uptake occurs and lower in the more distal parts, there probably enabling the uptake of the remaining luminal glucose content.

Diabetes mellitus affecting small intestinal hexose transporter expression

Several studies show dietary effects on intestinal monosaccharide transporter mRNA expression [110]. Dietary fructose and sucrose indeed increased jejunal hexose transporter (including SGLT1, GLUT2 and GLUT5) expression in non-diabetic rats [17]. Whereas expression of the fructose transporter GLUT5 seems to be specifically responsive to fructose, intestinal expression of SGLT1 and GLUT2 also depends on dietary glucose [63]. The regulation of intestinal hexose transporter in diabetic subjects seems to be complex and elicited contradictory findings: Whereas one study in humans shows increased gene and protein expression of intestinal hexose transporters GLUT2, GLUT5 and SGLT1 in humans with fasting blood glucose levels being above 5.5 mM and/or random blood glucose concentrations above 10.5 mM [16], another study performed in Zucker rats – reflecting a model of type 2 diabetes – shows no effect on intestinal monosaccharide transporter GLUT2, GLUT5 and SGLT1 expression [20]. Surveys in rodents with streptozotocin-induced type 1 diabetes show increased small intestinal GLUT2 and SGLT1 expression [64,65] with inconsistent findings concerning small intestinal GLUT5 expression [63,65].

In the present study we show, that patients with diabetes mellitus type 2 (without metformin treatment) exhibit increased duodenal GLUT2 mRNA expression levels when compared to non-diabetic control patients. Similarly, but not statistically significant, also the luminal fructose transporter GLUT5 shows a tendency to elevated gene expression in diabetics (without metformin treatment) when compared to controls. Interestingly mRNA levels of the secondary active brush border membrane glucose and galactose transporter SGLT1 were not different between these 2 groups of patients. To the author’s knowledge, the study by Dyer

and co-workers [102] reflects the only investigation assessing small intestinal hexose transporter expression in human subjects suffering from type 2 diabetes mellitus. In comparison to our findings, Dyer and colleagues observed increased gene expression of all 3 hexose transporters GLUT2, GLUT5 and SGLT1 and raised protein expression levels of GLUT5 and SGLT1 in subjects with impaired fasting glucose (denominated 'diabetic' by Dyer et al.). This discrepancy might be explained by the fact, that Dyer and co-workers allocated patients with fasting blood glucose levels > 5.5 mM and/or random blood glucose concentrations > 10.5 mM to the 'diabetic' group, whereas in the present study, the current recommendations from the World Health Organization and the American Diabetes Association [58] (i.e. fasting plasma glucose levels ≥ 7.0 mmol/l (126 mg/dl) and/or $HBA_{1c}$ concentrations > 6.5%) were applied to assign patients to the 'diabetic' group. Furthermore, the results presented by Dyer and coworkers might also reflect in part the differential nutritional state of diabetics and controls, since the latter individuals consumed normal diets whereas diabetic patients were on reduced carbohydrate diets in this survey.

Most studies in rodents assessing the effect of experimental diabetes on intestinal hexose transporter expression were performed in animal models of type 1 diabetes mellitus (i.e. streptozotocin-induced) [64,65]. An exception constitutes the study by Corpe and co-workers using an animal model of type 2 diabetes mellitus (i.e. Zucker Diabetic Fatty Rats). Corpe et al. hereby found no difference in hexose transporters GLUT2, GLUT5 and SGLT1 gene and protein expression in diabetic rats when compared to non-diabetic control animals. It is questionable, whether the discrepancy between the findings by Corpe and collaborators and our results (and also those of Dyer et al.) might reflect species differences or potentially biasing (and unknown) influencing factors, that may not be controlled in a cohort of human subjects. It is further remarkable that the study by Dyer et al. – next to the present work – reflects the only known investigation testing the effect of T2DM on intestinal hexose transporters expression in humans. This indeed raises the suspicion of non-publication of negative results by others and could hence also reflect a possible publication bias [111].

Transcription factors reported to participate in the regulation of kidney GLUT2 in diabetic animals are hepatocyte nuclear factors 1α, 4α and 3β (HNF-1α, HNF-4α and HNF-3β) [112,113]. *Hence, the gene expression of these transcription factors was assessed, revealing…* (Remark: The results of these experiments were pending when the present thesis was written)

Currently, most patients suffering from diabetes mellitus type 2 are treated with the biguanide metformin that reflects the first-line pharmacotherapy for T2DM. As metformin treatment was shown to exert an inhibitory effect on intestinal glucose absorption in rodents [14] and inconsistent effects of metformin on intestinal monosaccharide transporter (SGLT1, GLUT2 and GLUT5) expression were found in non-diabetic rodents [61,62], we analyzed the diabetic patients under metformin treatment separately. Interestingly, the increase in duodenal GLUT2

gene expression and the tendency to elevated small intestinal GLUT5 mRNA in diabetic patients without metformin treatment was completely abolished in patients under biguanide therapy. This could signify, that – among other metformin actions (i.e. decreased hepatic glucose output, increased fat and muscle tissue glucose uptake, reduced fatty-acid oxidation and increased intestinal use of glucose [12,13]) – also the opposing effect of metformin to T2DM on small intestinal GLUT2 (and probably also GLUT5) transporter expression could be an additional mode of action of metformin therapy. In contrast to our findings, 8 of 25 patients in the diabetic group reported by Dyer et al. were under metformin treatment and no information was available for 5 of 25 diabetic patients, meaning that metformin treatment in about one third of patients did not mask the increase in small intestinal hexose transporter expression observed their study.

Taken together, our study indicatess that T2DM patients have an increased GLUT2 mRNA expression, possibly an increase of GLUT2 expression in the luminal enterocyte membrane, as well as a non-significant increase in GLUT5 transcript expression, and that these effects are reversible under metformin. Since an increase in low affinity luminal diffusion pathways such as the GLUT hexose transporters presumably leads to accelerated luminal hexose absorption, this will also lead to stronger postprandial (or post softdrink) hyperglycemia. In view of the fact that such glucose excursions are considered to account for a large fraction of the HbA1c[114] increase and that they additionally represent an independent risk factor for adverse cardiovascular outcomes[115,116], it appears that the reported effects of T2DM on GLUT expression and its reversibility under metformin may play an important pathophysiological role.

**Figure legends**

**Figure 1**. **Relative mRNA abundance of hexose transporters along the human intestine.** The gene expression levels of GLUT2, GLUT5 and SGLT1 in 10 patients with combined gastroduodeno- and ileocolonoscopy are shown. The bars indicate mean relative mRNA expression ± SEM (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)). Gene expression of each transporter is shown at four different intestinal localizations: duodenum, part II (white bars), part III (light grey bars), terminal ileum (dark grey bars) and ascending colon (black bars).

**Figure 2. Effect of diabetes mellitus type 2 with and without metformin-treatment on small intestinal hexose transporter expression.** Duodenal mRNA expression (mean ± SEM) of monosaccharide transporters GLUT2 (**A**), GLUT5 (**B**) and SGLT1 (**C**) (normalized to villin ($2^{Ct(Villin)-Ct(target)}$)) in type 2 diabetic patients under metformin treatment (T2DM + metformin; black bars; n= 10) vs. diabetic patients without metformin therapy (T2DM – metformin; grey bars; n= 5) vs. non-diabetic control subjects (controls; white bars; n= 31) is shown. **, $p< 0.01$, ANOVA with post-hoc Bonferroni's multiple comparison test for all pairs of columns.

**Figure 3. Immuno-localization of GLUT5 in human intestine.** Representative tissue specimens from duodenum part II (**A**), duodenum part III (**B**), terminal ileum (**C**) and ascending colon (**D**) are shown. GLUT5 (green) localizes tot he brush border membrane of small intestinal enterocytes (**A-C**) and is not expressed in the large intestine (**D**). Cellular DNA (lower line; DAPI) is shown in blue to display the nuclei. Pictures were taken at 20x magnification. The white bar represents 100µm.

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**Tables and Figures**

**Table 1. Patient's data.**

| | Control (n=31) | DM II + metformin (n=10) | DM II, no metformin (n=5) | C vs. D+M | C vs. D-M | D+M vs. D-M |
|---|---|---|---|---|---|---|
| Gender (f/m) | 19/12 | 5/5 | 2/3 | n. a. | n. a. | n. a. |
| Age [years] | 56.2 ± 2.4 | 61.4 ± 4.0 | 59.6 ± 4.0 | n. s. | n. s. | n. s. |
| BMI [m/kg$^2$] | 25.2 ± 0.9 | 26.6 ± 1.3 | 28.6 ± 0.6 | n. s. | n. s. | n. s. |
| Glucose [mmol/l] | 6.2 ± 0.2 | 9.8 ± 1.1 | 7.1 ± 0.4 | *** | n. s. | * |
| HbA1c (NGSP) [%] | 5.8 ± 0.06 | 7.1 ± 0.5 | 6.6 ± 0.1 | *** | n. s. | n. s. |
| Creatinine [µmol/l] | 74.2 ± 2.6 | 64.7 ± 4.7 | 80.0 ± 10.2 | n. s. | n. s. | n. s. |
| Urea [mg/dl] | 4.5 ± 0.2 | 4.7 ± 0.4 | 5.9 ± 0.5 | n. s. | * | n. s. |

**Table 1.** Control; C; Control patients. DMII; Diabetes mellitus type II. DM II + Metformin; D+M; Diabetes mellitus type II treated with metformin. DM II, no metformin; D-M; Diabetes mellitus type II without metformin treatment. BMI; body mass index. N. s.; not significant; $p > 0.05$. N. a.; not assessed. *; $p < 0.05$. **; $p < 0.01$. ***; $p < 0.0001$. ANOVA with post-hoc Bonferroni's Multiple Comparison test.

**Table 2. Primers and Probes used for Real-time PCR.**

| Protein/Gene | Type | Sequence |
|---|---|---|
| GLUT2/SLC2A2 | Forward primer | AGC ATT TTT CAG ACG GCT GG |
| | Reverse primer | TAG AAA GAG AGA ACG TCG CCC |
| | Probe | ACC TGT TTA TGC AAC CAT TGG AGT TGG C |
| GLUT5/SLC2A5 | Forward primer | TCA TTG TCT TCG CCG TGA TC |
| | Reverse primer | TCT ATG AAC GTC TTG GCC TTG |
| | Probe | CTC CTC ACC ACC ATC TAC ATC TTC TTG ATT GTC C |
| SGLT1/SLC5A1 | Forward primer | TGC CGG AAG GTT GTT TAT CC |
| | Reverse primer | GTT GCC CAC TTT GTG CTG ACT |
| | Probe | CAT CAG CAT CGC CTG GGT GCC |
| Villin | Forward primer | AGG ATG ATG TGT TCC TAC TAG ATG TCT G |
| | Reverse Primer | GTT TCT GCG GCC TTC TTC |
| | Probe | TGT TTC CCA ATC CAG AAG AAG ACC TGG TC |

**Figure 1**

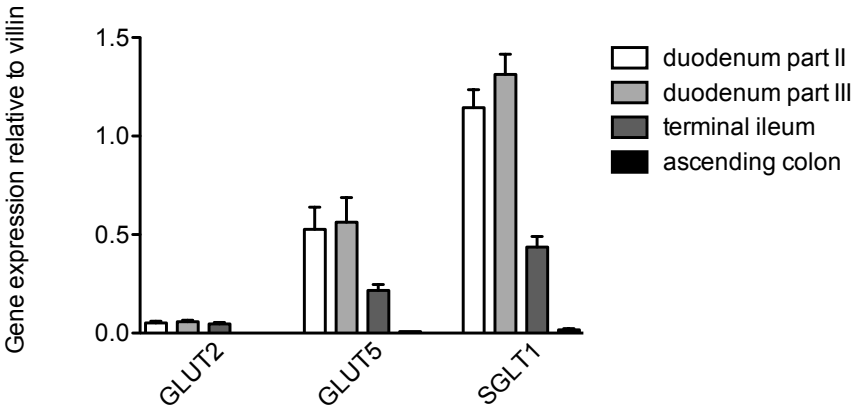

Gene expression relative to villin
1.5
1.0
0.5
0.0
GLUT2
GLUT5
SGLT1
duodenum part II
duodenum part III
terminal ileum
ascending colon

**Figure 2**

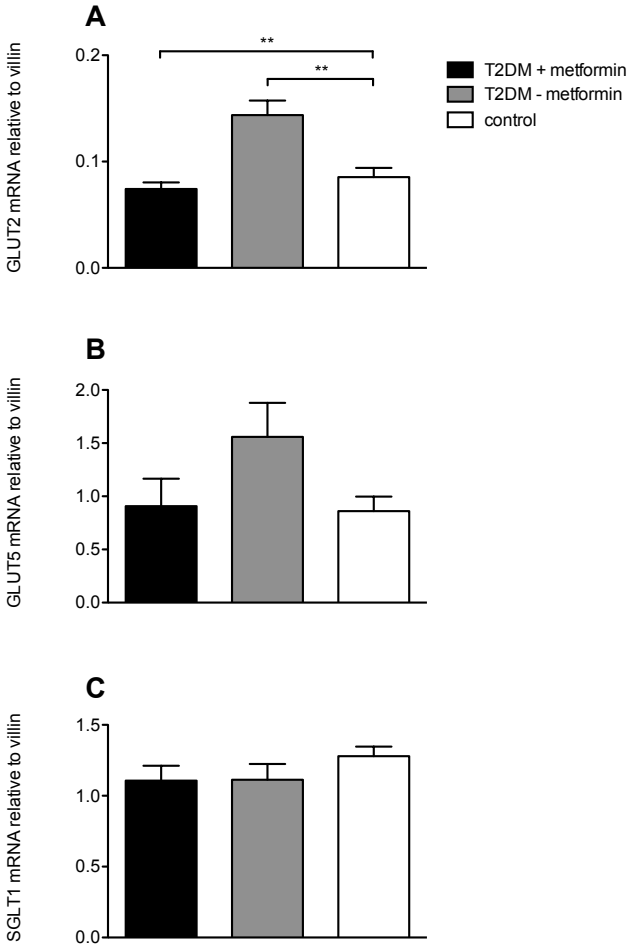
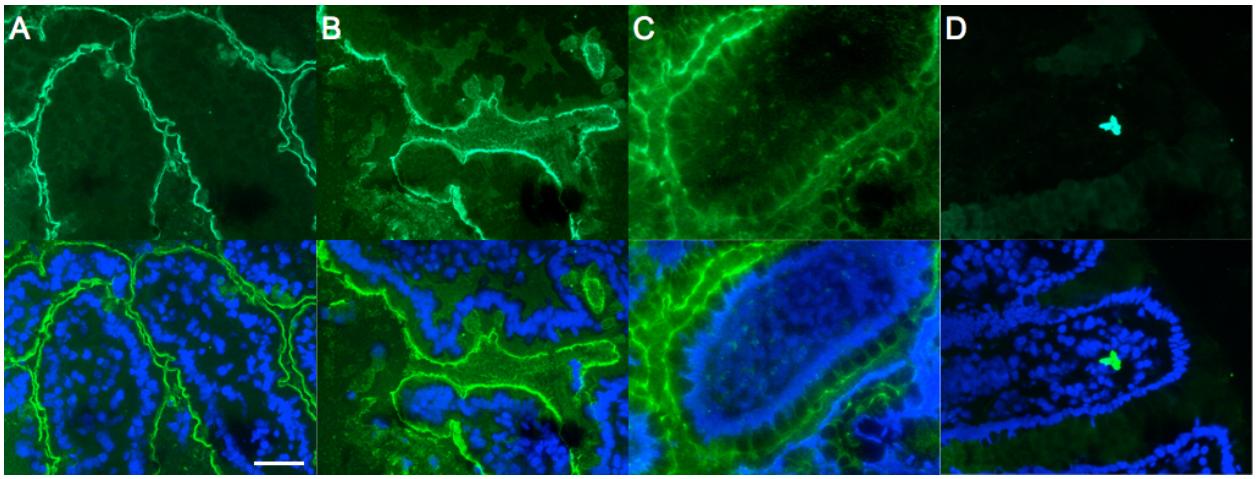

A
B
C
GLUT2 mRNA relative to villin
GLUT5 mRNA relative to villin
SGLT1 mRNA relative to villin
0.0
0.1
0.2
0.5
1.0
1.5
2.0
**
**
T2DM + metformin
T2DM - metformin
control

**Figure 3**



# 7. Manuscript: 'The molecular mechanism of intestinal levodopa absorption and its possible implication on treatment of Parkinson's disease'

This section contains the manuscript *'The molecular mechanism of intestinal levodopa absorption and its implications on treatment of Parkinson's disease'.*

Dr. Simone M. R. Camargo significantly contributed to this research article and performed the experiments in rodents and in cell culture.

My contribution to the manuscript concerned the creation of the fusion protein human $b^{0,+}$AT – human rBAT and the experiments performed in *Xenopus laevis* oocytes.

**The molecular mechanism of intestinal levodopa absorption and its possible implication on treatment of Parkinson's disease.**

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Running title: levodopa transport in intestine

***Keywords***

$b^{0,+}$AT-rBAT, SLC7A9; $B^0$AT1, SLC6A19; SIT1, SLC6A20; ACE2; levodopa; carbidopa; entacapone; bensirazide; Parkinson's disease

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**Abstract**

Levodopa (L-DOPA) is the naturally occurring precursor amino acid of dopamine and the main therapeutic agent for neurologic disorders of dopamine depletion, such as Parkinson's disease. Levodopa absorption in small intestine has been suggested to be mediated by the large neutral amino acids transport machinery, but the identity of the involved transporters is unknown. Clinically, co-administration of levodopa and dietary amino acids is avoided to decrease competition for transport in intestine and at the blood brain barrier. Another common practice is the co-administration of levodopa metabolism inhibitors (dopa decarboxylase and cathechol-o-methyl transferase inhibitors), which also share structural similarity with levodopa. In this systematic study involving *Xenopus laevis* oocytes and MDCK epithelia as expression systems and *ex vivo* preparations from wild type and knock out mice we identified the neutral and dibasic AA exchanger (antiporter) $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) as the luminal intestinal levodopa transporter whereas the major luminal cotransporter (symporter) $B^0$AT1 (SLC6A19) was not involved. L-leucine and L-arginine competed with levodopa for transport across the luminal enterocyte membrane as expected for $b^{0,+}$AT-rBAT substrates whereas dopa decarboxylase and cathechol-o-methyl transferase inhibitors had no effect. The presence of amino acids in the basolateral compartment mimicking the postprandial phase increased transepithelial levodopa transport by stimulating its basolateral efflux via antiporter LAT2-4F2 (SLC7A8-SLC3A2). Additionally, the aromatic amino acid uniporter TAT1 (SLC16A10) was shown to play a major role. These results identify the molecular mechanisms mediating small intestinal levodopa absorption and might lead to optimization of strategies for delivery and absorption of this important pro-drug.

**Introduction**

Parkinson's disease (PD) [117] is a neurodegenerative disorder mainly caused by dopamine depletion in the substantia nigra, clinically manifesting by different symptoms including its hallmark, the trias of bradykinesia, resting tremor and rigidity [23,118,119]. Since its introduction in 1968, levodopa (L-Dihydroxyphenylalanine, L-Dopa) is the major therapeutic agent in treating PD [120]. After passing the Blood Brain Barrier (BBB), levodopa is converted into dopamine by the dopa-decarbocylase (DDC) [23]. To prevent levodopa metabolism prior to its transport across the BBB, orally administered levodopa is given in combination with a DDC inhibitor (DDCI) (as e. g. carbidopa or benserazide) [22]. It may be additionally combined with a cathechol-o-methyltransferase (COMT) inhibitor (such as entacapone) to avoid levodopa methylation into 3-o-methyldopa.

Levodopa is a large neutral amino acid structurally very similarto the aromatic amino acids (AAs) L-phenylalanine (Phe) and L-tyrosine (Tyr). Levodopa is believed to compete with other neutral amino acids for its active transport across the blood-brain barrier, as well as across small intestinal enterocytes. In the brain, levodopa has been suggested to be transported by the neutral amino acid heterodimeric transporter LAT1-4F2hc (SLC7A5-SLC3A2) [118,121,122]. Intestinal levodopa transport has been also suggested to be mediated by neutral amino acid transporters [69], but the identity of the transporters involved in its intestinal absorption is still unknown. Several luminal and basolateral AA and peptide transporters located at the luminal and basolateral enterocyte membrane have been shown to be responsible for neutral AA absorption [1,38,123]. $B^0AT1$ (SLC6A19) is the major transporter for neutral amino acids in the apical enterocyte membrane [73,75,124,125]. The neutral and dibasic amino acid exchanger (antiporter) $b^{0,+}AT$-rBAT (SLC7A9-SLC3A1) is the transporter responsible for cystine reabsorption in the kidney, but as well cationic and neutral amino acids across the luminal enterocyte membrane [70,126-130]. Among the basolateral enterocyte transporters the AA exchangers $y^+LAT1$-4F2hc and LAT2-4F2hc (SLC7A8-SLC3A2) [131-133], and the aromatic AA uniporter TAT1 (SLC16A10) [26,79,93,134,135] were shown to promote accumulation or efflux of amino acid from the enterocytes to the extracellular space. In addition the functional interaction between different transporters can affect the net flux of amino acids [1,135,136].

In the present study, we first intended to identify the levodopa transporter(s) of the luminal membrane of small intestinal enterocytes. Several candidate transporters were identified by means of structural homology of their transported substrates to levodopa and tested. Second, the transepithelial transport involving apical and basolateral transporters in cells and along the murine small intestine was assessed using a polarized cell system and intestinal everted sacs. Additionally, the impact of luminal neutral amino acids (reflecting digested dietary proteins) and routinely co-administered DDCIs and COMT inhibitors on intestinal levodopa transport was evaluated as well the impact of gender and circadian rhythm on intestinal transporter expression. Concisely, the particular role of the apical $b^{0,+}AT1$-rBAT and basolateral TAT1 in intestinal levodopa transport was elucidated.

**Materials and Methods**

Oocytes

Expression studies and influx assays using radiolabeled amino acid tracers were performed in *Xenopus laevis* oocytes. The oocytes were injected with cRNA encoding transporters known or suggested to be expressed in the apical membrane of small intestinal enterocytes. $B^0$AT1-ACE2 (SLC6A19-*ace2*), SIT1-ACE2 (SLC6A20-*ace2*), PAT1 (SLC36A1), ASCT2 (SLC1A5) and $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1, fusion protein) cRNA respectively were therefore injected. The transport rate of levodopa (100 µM, 20 µCu / ml $^3$H-levodopa, containing 100 µM citric acid (in order to inhibit levodopa oxidation)) was measured and compared to specific substrate uptake rates for each transporter (as further described on the Figure legends). Data obtained for non-injected oocytes were subtracted, and levodopa uptake rates were normalized to those of the known substrates. Competition experiments of levodopa or leucine (100 µM) transport were conducted in the presence of 5 mM L-leucine or levodopa, respectively. For the DDC and COMT inhibitors, uptake was conducted with 1 µM levodopa (20 µCu / ml $^3$H-levodopa, 100 µM citric acid, for 1 minute) in the presence of 50 µM bensirazide, carbidopa or entacapone, respectively. For the main neutral amino acid transporter $B^0$AT1-ACE2, the transports of levodopa, L-leucine, L-phenyalanine, and L-tyrosine (1mM) were further measured using the two-microelectrode voltage clamp (TEVC) technique. Recordings were carried out as previously described [137] at a membrane holding potential (Vh) of −50 mV. Pooled data were shown as mean ± SEM with *n* representing the number of pooled cells.

Construction of the hrBAT-hb$^{0,+}$AT Fusion Protein

To assure $b^{0,+}$AT-rBAT transport analyzed in *X. laevis* oocytes was due to human protein, a fusion protein of human $b^{0,+}$AT and human rBAT was used. Briefly, we utilized the previously described fusion protein [130] and substituted mouse $b^{0,+}$AT by the human ortologue. Human $b^{0,+}$AT was amplified by PCR adding with XmaI at 5' and NsiI at 3' ( primer: sense': CAA AAC CCC GGG ATG GGG GAT ACT GGC CTG AGA AAG; antisense: CCG GAG GAA GAC CCT GAG GGT GCT GCT CCT GAT GGT GCT CCT GGA TGC ATT CAA). The encoding fragment was introduced in frame into the vector containing hrBAT and linker previously linearized using SmaI (compatible with XmaI) and NsiI. For expression in *Xenopus* oocytes the human $b^{0,+}$AT-rBAT-pSport plasmid was linearized with Hind III (Promega) and used as template for RNA synthesis from the T7 promoter (mMESSAGE mMACHINE, Ambion, Austin, Tex., USA).

Cells

MDCK (Madine Darby canine Kidney) cells (strain II) were cultured at 37°C in Dulbecco's modified Eagle's medium (Invitrogen, Basel, Switzerland) with 2 mM L-glutamine, 1% non-essential amino acids (Invitrogen) and 10% fetal calf serum (FCS). Cell lines previously

described by Bauch and collaborators [138] were used. A further transduction on these cells was performed as previously described to introduce the basolateral transporter TAT1 (SLC16A10). Four different cell lines were used: Wild type cells (1); cells expressing the apical transporter $b^{0,+}$AT-rBAT alone (2); cells expressing apical $b^{0,+}$AT-rBAT in the presence of the basolateral transporters LAT2-4F2hc (Slc7a8-Slc3a2) and y+LAT1-4F2hc (Slc7a7-Slc3a2) (3); and cells additionally (to cell line 3) expressing the basolateral uniporter TAT1 (4). The wild type and stably transfected cell lines were plated at density of 1.6 $x10^6$ cells / 24 mm Corning Costar Transwell filters and cultivated for 7 days. $b^{0,+}$AT-rBAT expression was induced 24h prior to the experiment with 1 µM dexamethasone. Integrity of the monolayers was checked by resistance measurements using the Millicell device (Millipore, Bedford, MA). At the experiment day, cells were incubated for 30 minutes with the dopa decarboxylase (DDC) inhibitor Bensirazide (50µM Bensirazide, 100 µM citric acid) in 150 mM NaCl, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$,5 mM KCl, 1 mM $MgCl_2$, 10mM glucose at 37°C. After this period, the uptake solution (100 µM levodopa, 50 µM Bensirazide, 100 µM citric acid) was added to both compartments. The filter compartment, facing the apical membrane of the cellular monolayer, was supplemented with 0.5 µCi/ml $^3$H-levodopa. In the competition experiments, L-leucine or L-lysine (1 mM) was additionally added to the apical compartment. To analyze the effect of basolateral amino acids on transepithelial levodopa transport (mimicking post-absorptive states with high plasma amino acid levels), L-leucine or L-lysine (1 mM) were added to the basolateral compartment.

After 30 minutes, apical and basolateral solutions were collected. Transwells were washed in both compartments with $Na^+$-free buffer at 4°C. Cells were disrupted by incubation with $Na^+$-deoxycholate and rocking for 30 minutes. After neutralizing with 0.1 M HCl, cells were added to vials and supplemented with a scintillation solution (Perking Elmer). The solution facing the cells in the filter compartment and the solution in the basolateral compartment were measured the same way. Pooled data are shown as mean ± SEM with *n* representing the number of filters used. For each experiment the values for wild type cells were subtracted from the overexpressing cells and data were expressed in nmol/hour/$cm^2$.

Animals

Wild type and *tat1* knockout mice were housed in standard conditions and fed a standard diet. A *tat1* knockout mouse model was produced by the ENU (N-ethyl-N-nitrosurea) mutagenesis (Ingenium Pharmaceuticals AG, Germany). After 10 backcrossings in a C57Bl/6J background, the animals were used on the described experiments [93]. All procedures for mice handling were performed according to the Swiss Animal Welfare laws Zurich, Switzerland.

Intestinal ring uptake

Uptake of radiolabeled amino acids was performed as previously described [5,139] on duodenal, jejunal and ileal segments. Briefly, everted small intestinal segments were incubated in oxygenated (Oxycarbon) Krebs-Tris buffer (pH 7.4) containing 100 µM Levodopa (0.05 µCi 3H-L-levodopa/mL, 50µM Bensirazide, 100 µM citric acid), for 5 minutes at 37°C or

4°C. After washing the rings with ice cold buffer, the segments were dried at 55°C overnight on cellulose (Sartorius AG, Goettingen, Germany) and weighed. Segments were then lysed in 0.75N NaOH for 6 hours and neutralized with 10N HCl, and the radioactivity was determined by liquid scintillation. Amino acid transport was expressed as pmol/mg/5min of dry tissue weight. Values obtained at 4°C were subtracted from the values of samples assayed at 37°C.

Everted intestinal sacs

In order to analyze transepithelial levodopa transport ex-*vivo*, everted jejunal sacs from wild type or *tat1* knockout animals were used. Everted sacs were built by constraining the extremities of circa 2 cm everted segments with a suture. The sacs were filled with Krebs-Tris buffer (pH 7.4) and incubated in a solution containing levodopa (100 µM levodopa, 0.05 µCi 3H-levodopa/mL) for 10 minutes. After washing with ice-cold solution, the liquid content of the sacs (serosa) was collected, the tissue (mucosa) was weight and processed as the intestinal rings. The data are shown as mean ± SEM with *n* representing the number of sacs. The transport is expressed as nmol/10 minutes/mg of dry tissue. Values obtained at 37°C were subtracted from measurements performed at 4°C.

Brush border membrane vesicles (BBMV) and Western blot analysis

BBMV were prepared from small intestine mucosal cells using the $Mg^{2+}$ precipitation technique as described elsewhere [140]. Western blotting was performed as previously described [138]. Primary antibodies used were: rabbit affinity purified anti-mouse $b^{0,+}$AT1 (diluted 1:500) and monoclonal anti-mouse actin (1:10'000) (Sigma, St Louis, MO). Secondary antibodies: donkey anti-rabbit IgG horseradish peroxidase conjugated (Amersham Biosciences, Piscataway, NJ) or anti-mouse IgG alkaline phosphatase conjugate (Promega, Madison, WI) were diluted 1:5'000. Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, MA) and chemiluminescence visualized with a DIANA III camera (Raytest, Dietikon, Switzerland).

Quantitative real time PCR (qPCR)

RNA was extracted from small intestine scraped mucosa, and real time quantitative reverse transcription-PCR was performed as previously described [75]. The abundance of the target mRNAs was calculated relative to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT). Relative expression ratios were calculated as $r = 2^{(Ct(reference) - Ct(test))}$, whit *Ct* representing cycle number at the threshold, and *test* representing tested mRNAs. Primers and probes used have been previously described [70,75,141].

Data analysis

Data are shown as mean ± SEM. All assessed data were unpaired and parametric. Differences between one 'control group' and two or more 'treatment groups' were assessed using an ANOVA with post-hoc Dunnett's Multiple Comparison test and differences between three or more groups (executing all possible comparisons between groups) were measured utilizing an ANOVA with post-hoc Bonferroni's Multiple Comparison test. For single pairwise comparisons, a Student's two-tailed *t*-test was performed. P-values of less than 5% were

considered significant. Graphs, descriptive statistics and calculations were performed using the software GraphPad Prism version 4.0 (GraphPad, San Diego, Calif., USA).

**Results**

***Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT but not by the major neutral amino acid transporter $B^0$AT1-ACE2***

To determine the apical amino acid transporter(s) responsible for levodopa transport across the luminal enterocyte membrane, candidate transporters were expressed in *X. laevis* oocytes. The uptake of levodopa was compared to known substrates (control amino acids) for each given transporter. As depicted on Figure 1A, oocytes expressing the amino acid transporters ASCT2 (SLC1A5), SIT1 (SLC6A20) and PAT1 (SLC36A1) did not transport levodopa. Oocytes expressing the AA exchanger $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) transported levodopa as efficiently as the control amino acid (L-leucine) used. The major neutral luminal AA transporter $B^0$AT1-ACE2 (SLC6A19-*ace2*) did not transport levodopa. The current generated by the co-transport of sodium (Figure 1B) and the accumulation of radiolabeled levodopa (Figure 1C) were negligible, despite structural homology of levodopa and $B^0$AT1 substrates L-phenylalanine and L-tyrosine. $b^{0,+}$AT-rBAT hence is the only known luminal levodopa transporter of small intestine.

***The transport of levodopa by $b^{0,+}$AT-rBAT can be inhibited by the presence of luminal neutral amino acids, but not by carbidopa, entacapone or benserazide.***

To test whether levodopa and the neutral amino acid L-leucine compete for $b^{0,+}$AT1-rBAT mediated transport, radiolabeled levodopa influx experiments in *X. laevis* oocytes were performed in the presence or absence of L-leucine and vice versa. Both, radiolabeled levodopa and L-leucine uptake was abolished by the presence of excess of non-labeled L-leucine and levodopa, respectively (Figure 2A).

Since DDCIs bensirazide, carbidopa and the COMT inhibitor entacapone display a similar structure to levodopa, a potential effect of these substrates on $b^{0,+}$AT1-rBAT-mediated levodopa transport was tested. The presence of bensirazide, carbidopa or entacapone did not affect levodopa transport (Figure 2B).

To analyze the impact of high intracellular neutral amino acid concentrations (mimicking the (post-)absorptive state) on AA uptake via the antiporter $b^{0,+}$AT-rBAT, uptake rates of levodopa and L-leucine, were measured after pre-injecting *X. laevis* oocytes with either L-arginine (12.5mM) or water (negative control). Pre-loading cells with L-arginine resulted in a small but non-significant rise of levodopa and L-leucine uptake (Figure 2C).

***Transepithelial transport of levodopa depends on expression of $b^{0,+}$AT-rBAT in the apical- and LAT2-4F2hc and TAT1 in the basolateral membrane***

To analyze the interaction of apical and basolateral membrane AA transporters on transepithelial levodopa transport, mammalian MDCK epithelial cell models overexpressing various combinations of heterodimeric amino acid antiporter and TAT1 uniporter was used [Ref Bauch]. Cells overexpressing only $b^{0,+}$AT-rBAT accumulated levodopa intracellularly, and showed – to some extent – a basolateral levodopa efflux (Figure 3A, white bars). Cells additionally expressing the antiporter LAT2-4F2hc in the basolateral membrane showed a 3- and 2-fold increase in intracellular levodopa accumulation and transepithelial levodopa transport-, respectively (Figure 3A, gray bars). Maximal transepithelial transport was observed in cells additionally overexpressing the facilitated diffusion protein TAT1 in their basolateral membrane. In these cells, a 5-fold increase in basolateral- and 3-fold decrease in intracellular accumulation was observed (Figure 3A, black bars).

To mimic the absorptive (presence of high amino acid concentrations in the lumen) or post-absorptive (presence of high amino acid concentrations in the extracellular space) state, excess concentrations of L-leucine or L-lysine were respectively added to the apical or basolateral compartments. The presence of excess L-leucine (Figure 3B) or L-lysine (Figure 3C) in the apical compartment reduced intracellular levodopa accumulation, as well as the transepithelial transport in all cell lines used. These results suggest that both L-lysine and L-leucine compete with levodopa, as expected for transport via $b^{0,+}$AT-rBAT.

Adding excess L-leucine to the basolateral compartment increased transepithelial transport of levodopa in cells expressing LAT2-4F2hc (Figure 3D, gray bars). These result suggests basolateral L-leucine stimulated the antiporter LAT2-4F2hc to export intracellularly accumulated levodopa to the basolateral compartment. To test whether $y^+$LAT1-4F2hc could also be involved in basolateral levodopa efflux, we analyzed the influence of the $y^+$LAT1 substrate lysine. The presence of excess L-lysine in the basolateral compartment had no effect on levodopa transport (Figure 3E), suggesting it does not transstimulated the efflux of intracellularly accumulated levodopa via antiporter $y^+$LAT1. Based on these results we suggest that LAT2-4F2hc, but not $y^+$LAT1, participates in basolateral levodopa efflux.

***Neutral and cationic amino acids compete with levodopa for its absorption in the murine small intestine.***

Levodopa accumulation in intestinal enterocytes was tested using mouse everted rings of different small intestinal segments (duodenum, jejunum and ileum). Cellular uptake was similar in all three intestinal segments in the condition tested (Figure 4A) and uptake-rates were therefore pooled for the competition experiments depicted in Figure 4B. Adding excess non-labeled cationic AA L-arginine or neutral AA L-leucine to the uptake solution reduced intestinal levodopa uptake (Figure 4B), similarly as observed in the cell culture (Figure 3B, C) or in *Xenopus laevis* oocytes (Figure 2B) for transport via $b^{0,+}$AT-rBAT.

***Basolateral uniporter TAT1 affects transepithelial levodopa transport in vivo***

To analyze the role of the basolateral AA uniporter TAT1 on levodopa efflux from mucosal cells into the serosal compartment and on its transepithelial transport, levodopa accumulation was measured using everted intestinal sacs. Intracellular levodopa accumulation in *tat1* (*slc16a10*) knockout mice did not differ from wild type animals (Figure 4C), whereas transepithelial levodopa transport was significantly reduced in preparation made from knock-out animals when compared to wild type mice (Figure 4D). These results suggest the aromatic amino acid transporter TAT1 to play a major role mediating levodopa efflux from intestinal enterocytes.

***Circadian and gender influence on transporters involved in levodopa absorption.***

Absorption of levodopa in humans follows a circadian rhythm with faster absorption during daytime and delayed absorption during night [142]. Furthermore, gender differences in levodopa bioavailability and clinical presentation were observed [143] [144]. We hence analyzed the expression of transporters involved in levodopa transport in the small intestine of male and female rodents during the active (related to daytime in humans) and non-active phase (related to nighttime in humans). Intestinal gene expression of transporters involved in levodopa absorption ($b^{0,+}$AT-rBAT, LAT2-4F2hc and TAT1) were not different between male and female mice and during active and non-active phase along the small intestine (Figure 5A). The RNA expression of $b^{0,+}$AT-rBAT was not different in males and females or during the different activity periods (Figure 5A), while protein expression of $b^{0,+}$AT showed a tendency to higher levels during the active phase in male animals when compared to female rodents, but did not differ among groups during the non-active period (Figure 5B).

## Discussion

In this study we identify the molecular mechanism involved in intestinal levodopa absorption. Levodopa is the main therapeutic agent used to treat Parkinson's disease. It structurally belongs to the class of large neutral amino acids, which also includes L-tyrosine, L-phenylalanine, L-tryptophan, L-leucine, L-isoleucine (Ile) and L-valine [145]. For more than 20 years it was suggested that levodopa absorption should take place by the same active transporters as large neutral amino acids, but the identity of the transporter(s) was not known [146]. By using several experimental models, we could show that $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1, apical), LAT2-4F2hc (SLC7A8-SLC3A2, basolateral) and TAT1 (SLC16A10, basolateral) are the amino acid transporters responsible for small intestinal levodopa absorption.

### *Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT but not by the major neutral amino acid transporter $B^0$AT1-ACE2*

In the blood brain barrier (BBB), levodopa was shown to be actively transported by the neutral amino acid antiporter LAT1-4F2hc [122]. In the kidney, its apical transport was suggested to involve the antiporter $b^{0,+}$AT-rBAT [134,147]. Several different AA transporters for neutral amino acids are expressed in the apical membrane of small intestinal enterocytes [1] and might therefore likewise transport levodopa. The broad neutral AA transporter $B^0$AT1 (SLC6A19) is the main luminal neutral amino acid transporter in the intestine and a likely candidate. $B^0$AT1 transports amino acids with close structural homology to levodopa (including in particular the aromatic amino acids Phe and Tyr). The IMINO transporter SIT1 (SLC6A20) and the proton-dependent amino acid transporter PAT1 (SLC36A1) represent other AA transporters of the apical enterocyte membrane. Both transporters to some extent also transport neutral amino acids [87,148]. The amino acid exchanger ASCT2 (SLC1A5) transports neutral amino acids as well, and was suggested to be expressed at the apical membrane of small intestinal enterocytes [149]. More recent data suggest however that it localizes rather to the basolateral membrane [.....Borer]. We thus assayed the potential transport of levodopa by these different known or predicted luminal AA transporters, specifically $B^0$AT1, SIT1 (SLC6A20), PAT1 (SLC36A1), ASCT2 (SLC1A5) and $b^{0,+}$AT-rBAT. With the exception of the cells expressing the antiporter $b^{0,+}$AT-rBAT, other transporters listed here did not show levodopa transport (Figure 1). Despite the fact that the main neutral amino acid transporter $B^0$AT1-ACE2 transports large neutral amino acids (Tyr and Phe) whose structure differ from levodopa only by the presence of less hydroxyl groups on the aromatic ring, oocytes expressing $B^0$AT1 did not show levodopa transport as evidenced by two-electrode voltage clamp or accumulation of radiolabeled AA. The hydroxyl group substitution seems to reduce $B^0$AT1 affinity to the substrate, but had no effect on $b^{0,+}$AT-rBAT mediated transport. $b^{0,+}$AT-rBAT transports Phe, Leu, Tyr, as well as methionine, glutamine, histidine, ornithine, cationic amino acids and the di-peptide cystine [126,130,150]. Furthermore, the

transporter $b^{0,+}$AT-rBAT was also previously shown to be involved in intestinal drug (gabapentin) absorption in rodents [136] and renal levodopa re-absorption [134]. It suggests a less rigorous selectivity of the transporter by accepting diverse small molecule structures. This broad substrate selectivity makes luminal intestinal and proximal tubule transporter $b^{0,+}$AT-rBAT an interesting pharmacological target. Broad selectivity was also observed for the basolateral antiporter LAT2-4F2hc and by the BBB levodopa transporter LAT1-4F2hc. LAT2-4F2hc was shown to transports a broad range of proteinogenic neutral amino acids, and additionally accepted levodopa, 3-o-methyldopa, T3 and T4 hormones. LAT1-4F2hc was shown to accept levodopa, 3-o-methyldopa, α-methylphenylalanine, α-methyltyrosine, α-methyldopa, phenylalanine methylester (Phe-Me), T3 hormone and gabapentin [121]. Interestingly, dopamine formed by the metabolism of levodopa is not transported by $b^{0,+}$AT-rBAT [134], LAT2-4F2hc [121], LAT1-4F2hc, TAT1 [79], or $B^0$AT1-ACE2 [137]. Different substrates (i.e. amino acids) compete according to their affinity and local concentration for their transporter. In humans' small intestine, the co-administration of Leu was shown to decrease the absorption of levodopa [69], an effect that might be explained by competition of these two amino acids for transepithelial transport. In our study we reveal the molecular mechanism of this competition in the small intestine. By using oocytes and cell cultures overexpressing $b^{0,+}$AT-rBAT we could demonstrate that Leu or cationic amino acids inhibit levodopa transport by competing for the apical transporter $b^{0,+}$AT-rBAT (Figures 2 and 3). Furthermore, Leu and cationic amino acids competed with levodopa along the mouse small intestine (Figure 4), similarly as reported in humans. The uptake of levodopa was comparable in the three segments of mouse small intestine (Figure 5A), even though we observed higher expression of $b^{0,+}$AT RNA and protein levels towards the ileum, as shown previously [70]. In humans, we also observed that $b^{0,+}$AT (SLC7A9) gene expression increases towards the more distal segments of the small intestine (Vuille-dit-Bille et al., unpublished data). The fact that gene/protein expression and absorption levels did not correlate might be explained by other influencing factors, including gastrointestinal motility, gastric secretion and gastric emptying [1,123,151,152].

Taken together, $b^{0,+}$AT-rBAT appears to be the only luminal enterocyte levodopa transporter, and its transport is competitively inhibited by luminal neutral- and dibasic amino acids.

Levodopa is metabolized to dopamine by the dopamine decarboxylase in different tissues, including brain, kidney and intestine [134,147]. To prevent levodopa metabolism prior to its transport across the BBB, it is usually administered in combination with a DDCI such as carbidopa or bensirazide. Further inhibitors of levodopa metabolism, such as the COMT inhibitor entacapone may be included in the formulation [22]. The transport mechanism of these substrates in the small intestine is not known. Carbidopa is not transported by LAT1-4F2hc and LAT2-4F2hc [121,122], however, a possible effect of carbidopa, bensirizide or entacapone on $b^{0,+}$AT-rBAT transport were not yet assayed. Despite the fact that carbidopa, entacapone or benserazide have some structural similarity to levodopa, no inhibition of

levodopa transport in *X. laevis* oocytes expressing $b^{0,+}$AT-rBAT was observed (Figure 2b). We thereof conclude that levodopa transport across the luminal enterocyte membrane mediated by $b^{0,+}$AT-rBAT is not affected by the co-administration of carbidopa, entacapone or benserazide.

Since $b^{0,+}$AT-rBAT is an obligatory amino acid exchanger (antiporter), it was suggested that preloading cells with amino acids could increase levodopa uptake. Hence, $b^{0,+}$AT-rBAT was shown to functionally interact with the peptide transporter PEPT1 (Slc15a1). Peptides transported inside the cell by PEPT1 and subsequently metabolized to single amino acids could be used by $b^{0,+}$AT-rBAT as exchanger substrates (*trans-stimulation)*. Nguyen and colleagues [136] showed that the increased amount of intracellular neutral amino acids following PEPT1 mediated peptide uptake and intracellular metabolism to single amino acids increased the entry of gabapentin mediated by $b^{0,+}$AT-rBAT, resulting in increased accumulation of the drug in rat jejunum. Using *X. laevis* oocytes the intracellular concentration of amino acids can be directly manipulated, but preloading oocytes with the cationic amino acid L-arginine showed only a trend to increased L-leucine and levodopa uptake (Figure 2C) that was not statistically significant. The functional interaction of transporters may be more complex and other intracellular amino acids may be involved as shown for LAT1-4F2hc and ASCT2 functional interaction couples [153]. Important to notice, that the higher intracellular concentration of the cationic $b^{0,+}$AT-rBAT substrate Arg did not inhibit the levodopa accumulation in cells. These results suggest that a preload of cells before the ingestion of levodopa may not be deleterious.

***Transepithelial transport of levodopa depends on LAT2-4F2hc and TAT1 expression in the basolateral membrane***

The basolateral large neutral amino acid antiporter LAT2-4F2hc (SLC7A8-SLC3A2) and the basolateral uniporter TAT1 (SLC16A10) [26,134,154] were previously assayed in overexpression systems for their ability to transport levodopa. In our epithelial cell culture model [Bauch et al.,], labeled levodopa was given to the luminal compartment, but the same concentration of unlabeled levodopa was also given in the basolateral compartments. Expression of the basolateral antiporter LAT2-4F2hc induced an increase in basolateral efflux of labeled levodopa. This suggests that intracellular levodopa was exchanged with extracellular one. The apparent affinity of LAT2-4F2hc for levodopa on the extracellular compartment was estimated to be in the micromolar range [154], whereas its intracellular affinity is not known, but may be lower as observed for other neutral amino acids [78]. The fact that additional high basolateral Leu (5 mM), but not Lys (5 mM), further transstimulated levodopa efflux ~2-foldindicates that 100 µM extracellular levodopa only approximately half-maximally activated LAT2-4F2hc function and thus suggests that 100 µM corresponds approximately to the $K_{0.5}$ of this transporter for levodopa. Interestingly, in cells expressing additionally TAT1, the same level of labeled levodopa efflux was observed in the absence of

high basolateral neutral amino acids. This may be explained by the fact that TAT1 is a uniporter (facilitated diffusion pathway) the bidirectional function of which does not depend on the presence of contralateral substrate [26,79]. Using this epithelial co-expression system we thus showed that both AA transporters LAT2-4F2hc and TAT1 can function as basolateral levodopa efflux pathways and consequently participate to its transepithelial transport. Interestingly, a functional interaction of these two basolateral enterocyte transporters was previously shown [135]. Similarly, as observed for PEPT1 and $b^{0,+}$AT-rBAT in the apical membrane, TAT1 can stimulate the function of the antiporter LAT2-4F2hc. For instance, aromatic amino acids exported by the uniporter TAT1 represent high affinity extracellular substrates for LAT2-4F2hc that can thus be exchanged by this antiporter with intracellular substrates like levodopa, thereby increasing trans-epithelial transport. This functional interaction is postulated to be crucial for the net directional transport of LAT2-4F2hc substrate amino acids and may thus influence the net levodopa flow. Ex-vivo experiments using everted gut sacs of *tat1* knockout animals confirmed that TAT1 plays a central role as basolateral efflux pathway for levodopa using experimental conditions without basolateral amino acids.

**Levodopa absorption along the small intestine and gender and circadian variation of transporters.**

Parkinson patients develop several non-motor symptoms and some have a diurnal component suggesting circadian dysfunction [155-157]. Gastrointestinal (GI) dysfunction is common in all stages of the disease. Delayed gastric emptying and constipation are the most common symptoms [158]. Delayed gastric emptying can influence the absorption of levodopa, causing wearing-off symptoms by inducing fluctuations in plasma levodopa levels. Orally administered levodopa is usually delivered throughout the day in several doses to avoid the wearing-off symptoms, and because the drug was shown to be absorbed faster during the active phase [142]. This difference in circadian pharmacokinetics may be a combination of the nocturnal delayed gastric emptying, supine position, and daily rhythmicity in gastrointestinal enzyme activity and blood flow. Circadian variations in function may be coordinated by a combination of a passive response to the daily cycle of food intake and endogenous timekeeping system [152]. Furthermore, gene expression levels of some small intestinal transporters have been shown to exhibit circadian rhythms. For instance, sugar transporters, including the apical $Na^+$ /glucose co-transporter 1 (SGLT1/Slc5a1) and fructose transporter 5 (GLUT5/Slc2a5) as well as the basolateral hexose transporter GLUT2/Slc2a2 were shown to have rhythmic expressions [101,159,160]. Additionally, the expression of amino acid transporters involved in drug absorption like the proton coupled peptide transporter 1 (PEPT1/Slc15a1) also shows a circadian expression cycle [101,160-162]. The mRNA expression levels of intestinal transporters $b^{0,+}$AT, rBAT, 4F2hc, LAT2 and TAT1 during the active (reflecting day-time expression in humans) and non-active (reflecting nocturnal gene expression in humans) phase of wild-type mice were not significantly different in the present study. The expression of the gene encoding the PEPT1 transporter, previously shown to

increase in the active phase [101], showed a similar tendency in our experiments that was however not statistically significant. In contrast, the protein expression level of the apical transporter $b^{0,+}$AT measured in brush border membrane vesicles was significantly increased during the active phase in male animals, but interestingly not in females. Since the changes in RNA levels precede the changes at the protein level, as shown for PEPT1 [162], we may have missed RNA changes that potentially took place earlier. Differences in the circadian cycle of male and females as observed in our study are not yet well understood [163], but importantly also in PD patients, gender differences have been indicated to play a major role. Men are more affected than women by the disease and its clinical symptoms and also onset and treatment outcomes differ [164,165]. Because in subjects receiving the same drug formulation a higher plasma concentration was found in females, it was suggested that the pharmacokinetics of levodopa would differ although in these studies the dose was not corrected to the body weight [144] [166] [167]. The higher plasma concentration in females could also be the result of differing metabolism or absorption, the latter parameter depending on gastric emptying and secretion, gastrointestinal motility, and transporter expression. The question of the influence solute transporter expression for the absorption of drugs is a growing field [168], but data related to gender differences are sparse.

Taken together, this study identifies the intestinal players involved in the absorption of levodopa, and detects $b^{0,+}$AT-rBAT as luminal enterocyte levodopa transporter. Moreover, this study also sheds light on the basolateral membrane with new information about the transporters involved in the second step of the transepithelial transport. The identification of the transporters involved in levodopa absorption, their mechanism of action, knowledge about trans-stimulation or competitive inhibition are very valuable information to optimize strategies for delivery and absorption of levodopa, the most important therapeutic agent in Parkinson's disease.

**Legends**

**Figure 1: Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT, but not by the major neutral amino acid transporter $B^0$AT1-ACE2. A) Oocytes expressing $b^{0,+}$AT-rBAT transport levodopa as efficiently as L-leucine.** Oocytes were injected with cRNA encoding mouse PAT1 (20ng), human SIT1 (5ng) and human accessory protein ACE2 (20 ng), human $B^0$AT1 (10 ng) and human accessory protein ACE2 (10ng), human ASCT2 (10 ng), or human $b^{0,+}$AT-rBAT fusion protein (10 ng). Levodopa uptake rates (100 µM; white bars) were compared to uptake rates of 100 µM L-glycine (PAT1), 100 µM L-proline (SIT1-ACE2), 100 µM L-leucine ($B^0$AT1-ACE2 and $b^{0,+}$AT-rBAT) and 100 µM L-alanine (ASCT2) (dark grey bars), respectively. The uptakes were performed at 25°C for 10 minutes. Results are expressed in pmol/oocyte/hour and values of non-injected oocytes were subtracted. Uptake results of control amino acids were normalized to 1. Bars represent means ± SEM, n= 4-9 injected oocytes per group. **, $p < 0.01$; ***, $p < 0.0001$; ns, not significant (unpaired two-tailed *t*-tests). **B and C) Oocytes expressing $B^0$AT1-ACE2 do not transport levodopa.** B) Levodopa does not induce current in oocytes expressing $B^0$AT1-ACE2: Oocytes expressing $B^0$AT1-ACE2 were superfused with L-leucine, L-phenylalanine L-tyrosine or levodopa (1 mM each). Results are expressed as current generated by the transport at a membrane potential of -50 mV and are represented as means ± SEM, n= 4 oocytes per group. *, $p < 0.05$ (ANOVA with post-hoc Dunnett's Multiple Comparison test). C) Levodopa is not accumulated in oocytes expressing $B^0$AT1-ACE2. The transport of L-leucine, L-phenylalanine, L-tyrosine and levodopa was assayed by measuring radiolabeled compound accumulation (1 mM, 30 minutes). Results are expressed in pmol/oocyte/hour and values of the non-injected oocytes were subtracted. Results are given as means ± SEM, n= 5-6 oocytes per group. *, $p < 0.05$ (ANOVA with post-hoc Dunnett's Multiple Comparison test).

**Figure 2: The transport of levodopa by $b^{0,+}$AT-rBAT can be inhibited by excess of neutral amino acids (and vice versa), but not by DDC inhibitors or COMT inhibitors. Transstimulating cells with L-arginine does not significantly increase levodopa uptake. A) Competitive inhibition of levodopa and L-leucine transport.** Radiolabeled levodopa (50 µM, white bars) and L-leucine (50 µM, black bars) uptake in *X. laevis* oocytes expressing $b^{0,+}$AT-rBAT was assayed in the presence or absence of excess non-labeled L-leucine (5mM) and levodopa (5mM), respectively. Results are expressed in pmol/oocyte/hour. Non-injected oocytes were subtracted. Results are expressed as mean ± SEM, n= 7-8 injected oocytes per group. **, $p < 0.01$; ***, $p < 0.0001$ (unpaired two-tailed *t*-tests). **B) DDC inhibitors bensirazide and carbidopa and the COMT inhibitor entacapone do not compete with $b^{0,+}$AT-rBAT mediated levodopa transport.** The transport of levodopa (1 µM / minute) was assayed alone (white bar), in the presence of bensirazide (50 µM, gray bar), carbidopa (50 µM, crosshatch bar), or entacapone (50 µM, black bar). Results are expressed in pmol/oocyte/hour and values of non-injected cells were subtracted. Data are represented as means ± SEM, n= 7-8 injected oocytes per group. The comparison of the 'treatment' groups

with the 'control' group (levodopa alone) yielded no differences (ANOVA with post-hoc Dunnett's Multiple Comparison test). **C) Intracellular L-arginine does not affected levodopa uptake into *X. laevis* oocytes expressing $b^{0,+}$AT-rBAT.** Oocytes were injected with the $b^{0,+}$AT-rBAT antiporter substrate L-arginine (final concentration in the oocyte achieved was 12.5 mM) or water (negative control). After 4 hours, the uptake with L-levodopa (50 µM; white bars) or L-leucine (50 µM; black bars) was performed. Data are represented as means ± SEM, n= 8-9 injected oocytes per experiment. No significant differences were seen (unpaired two-tailed *t*-tests; $p > 0.05$).

**Figure 3: Transepithelial transport of levodopa depends on $b^{0,+}$AT-rBAT expression in the apical and LAT2-4F2hc and TAT1 on the basolateral membrane.** MDCK cells expressing the apical transporter $b^{0,+}$AT-rBAT (white bars), the apical transporter $b^{0,+}$AT-rBAT and basolateral transporters LAT2-4F2hc and y+LAT1-4F2hc (gray bars) and (additionally to apical $b^{0,+}$AT-rBAT and basolateral LAT2-4F2hc and y+LAT1-4F2hc) TAT1 (black bars) were assayed in 5 different conditions (3A-E). **A)** Radiolabeled levodopa was added to the apical compartment. **B)**, Radiolabeled levodopa and L-leucine (5 mM) were added to the apical compartment. **C)** Radiolabeled levodopa and L-lysine (5 mM) were added to the apical compartment. **D)**, Radiolabeled levodopa in the apical compartment and L-leucine (5 mM) in the basolateral compartment. **E)**, Radiolabeled levodopa in the apical compartment and L-lysine (5 mM) in the basolateral compartment. In the conditions A-E both compartments contained solutions supplemented with 100 µM levodopa, 50 µM bensirazide, and 100 µM of ascorbic acid. The intracellular (middle panels) and the basolateral accumulation (right panels) of levodopa were measured and subtracted from the wild type cell values. Results are expressed in nmol/hour/$cm^2$ and given as mean ± SEM, n= 5-6 filters per experiment. *, $p < 0.05$ (ANOVA with post-hoc Bonferroni's Multiple Comparison test).

**Figure 4: Levodopa transport takes place all along the small intestine and can be inhibited by addition of luminal L-leucine and L-arginine or by ablation of basolateral transporter TAT1. A) Levodopa accumulates similarly in all segments of the small intestine.** Everted rings from duodenum, jejunum and ileum were incubated with levodopa (100 µM) at 37°C (white bars) or 4°C (crosshatched bar). Results are expressed in pmol/mg dry tissue/ 5min and given as nean ± SEM, n= 9-11 experiments per group. No significant differences between different intestinal localizations were seen (ANOVA with post-hoc Bonferroni's Multiple Comparison test; $p > 0.05$). **B) Neutral and cationic amino acids compete with levodopa transport.** Excess of L-leucine (1 mM, gray bar) and L-arginine (1 mM, black bar) were added to the uptake solution. Values obtained at 37°C were subtracted from uptakes performed at 4°C. Results are expressed in pmol/mg dry tissue/ 5min and given as mean ± SEM, n= 7-9 experiments per group. *, $p < 0.05$; **, $p < 0.01$ (ANOVA with post-hoc Dunnett's Multiple Comparison test). **C and D) The ablation of TAT1 decreases**

**transepithelial levodopa transport.** Everted jejunal sacs of wild type (black bars) and TAT1 knockout animals (gray bars) were assayed for levodopa transport. The accumulation in the tissue (mucosa) (**4C**) and the levodopa accumulation in the sacs resulting from the transepithelial transport (serosal) (**4D**) were quantified. Results are expressed in nmol/mg of dry tissue/ 10 minutes. Values at 4°C were subtracted and data are given as mean ± SEM, n= 11-12 everted sacs per group. Ns, not significant; *, $p < 0.05$ (unpaired two-tailed *t*-tests).

**Figure 5: Intestinal $b^{0,+}$AT-rBAT expression varies with gender and circadian rhythm at protein but not at RNA level. A) Expression of mRNA encoding the transporter $b^{0,+}$AT-rBAT (Slc7a9-Slc3a1), LAT2-4F2hc (Slc7a8-Slc3a2), TAT1 (Slc16a10) and peptide transporter PEPT1 (Slc15a1) in male and female animals during active and inactive periods were unchanged.** The gene expression of animals in the active (4 hours after the dark phase onset) and non-active phase (4 hours after the light phase onset) were analyzed by quantitative real time PCR. Results are expressed relative to the housekeeping gene HPRT and given as mean ± SEM, n= 3 female (solid bars) and 3 male (hatched bars) animals. ns, not significant; *, $p < 0.05$ (multiple two-tailed *t*-tests without applying a posttest to correct the global α-level). **B) The protein level of $b^{0,+}$AT in male animals is higher during the active phase when compared to the inactive phase.** B) Expression of $b^{0,+}$AT in the duodenum (white bars), jejunum (gray bars) and ileum (black bars) of female (solid bars) and male (hatched bars) mice in the active and inactive phase were analyzed by Western blot analysis of brush border membrane vesicles (50 µg). The Western blots were quantified by densitometry and normalized to beta actin. The results are expressed relative to actin and given as mean ± SEM, n= 3 animals each gender. ns, not significant; *, $p < 0.05$ (multiple two-tailed *t*-tests without applying a posttest to correct the global α-level).

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**Figures**

**Figure 1**

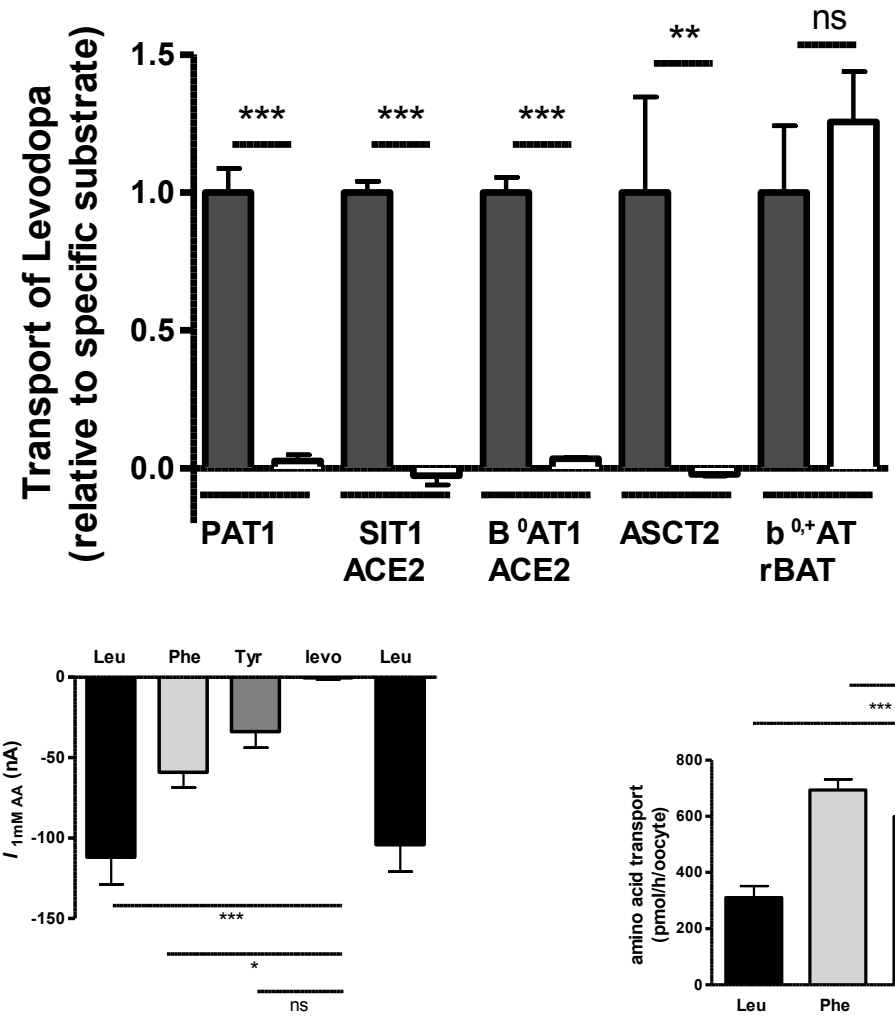
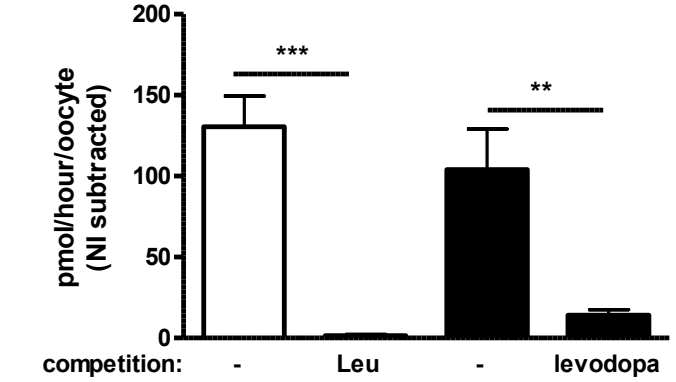
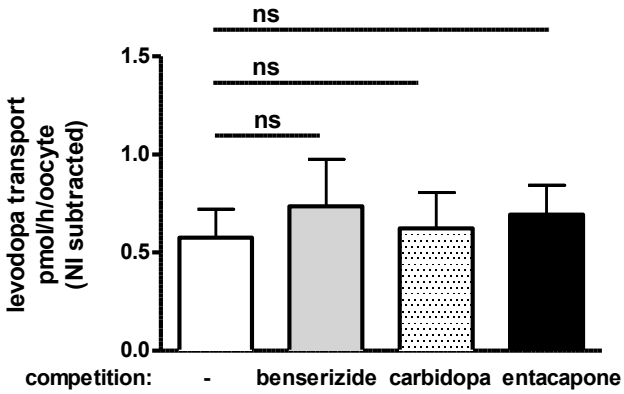


**Figure 2**


200
150
100
50
0
pmol/hour/oocyte
(NI subtracted)
***
**
competition:
-
Leu
-
levodopa


ns
ns
ns
1.5
1.0
0.5
0.0
levodopa transport
pmol/h/oocyte
(NI subtracted)
competition:
-
benserizide
carbidopa
entacapone

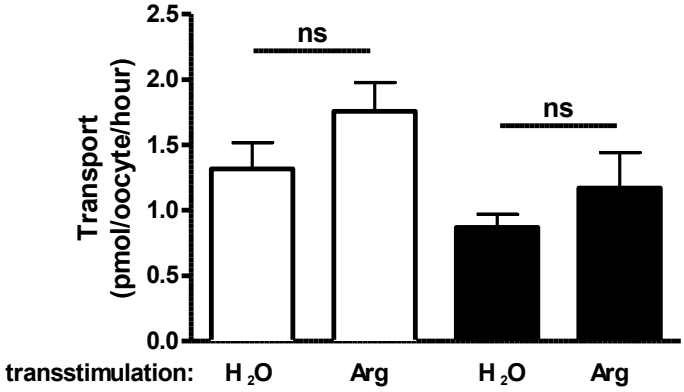

2.5
2.0
1.5
1.0
0.5
0.0
Transport
(pmol/oocyte/hour)
ns
ns
transstimulation:
H 2O
Arg
H 2O
Arg

**Figure 3**

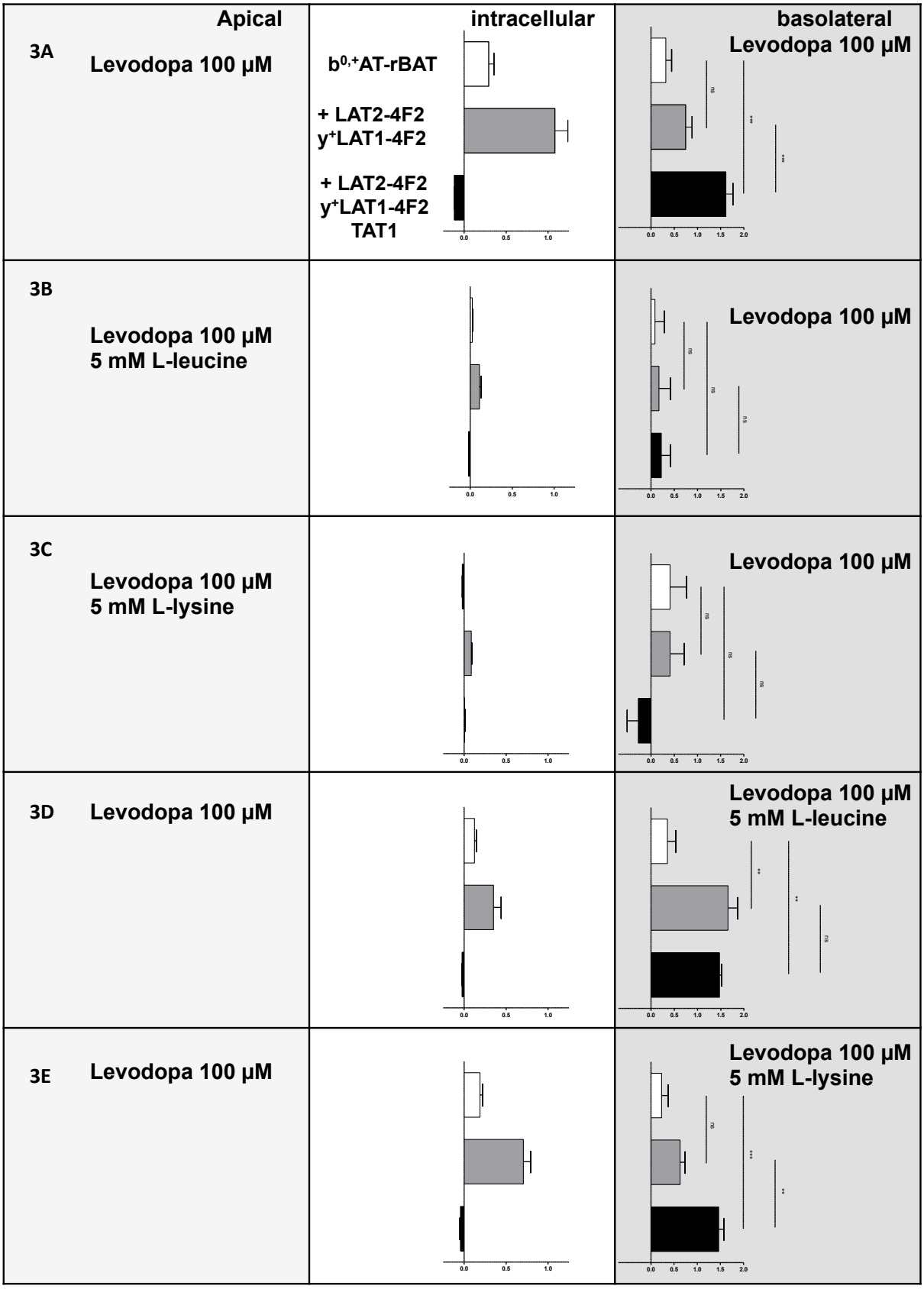

Apical
intracellular
basolateral
3A
Levodopa 100 µM
b0,+AT-rBAT
+ LAT2-4F2
y+LAT1-4F2
+ LAT2-4F2
y+LAT1-4F2
TAT1
Levodopa 100 µM
3B
Levodopa 100 µM
5 mM L-leucine
Levodopa 100 µM
3C
Levodopa 100 µM
5 mM L-lysine
Levodopa 100 µM
3D
Levodopa 100 µM
Levodopa 100 µM
5 mM L-leucine
3E
Levodopa 100 µM
Levodopa 100 µM
5 mM L-lysine

**Figure 4**

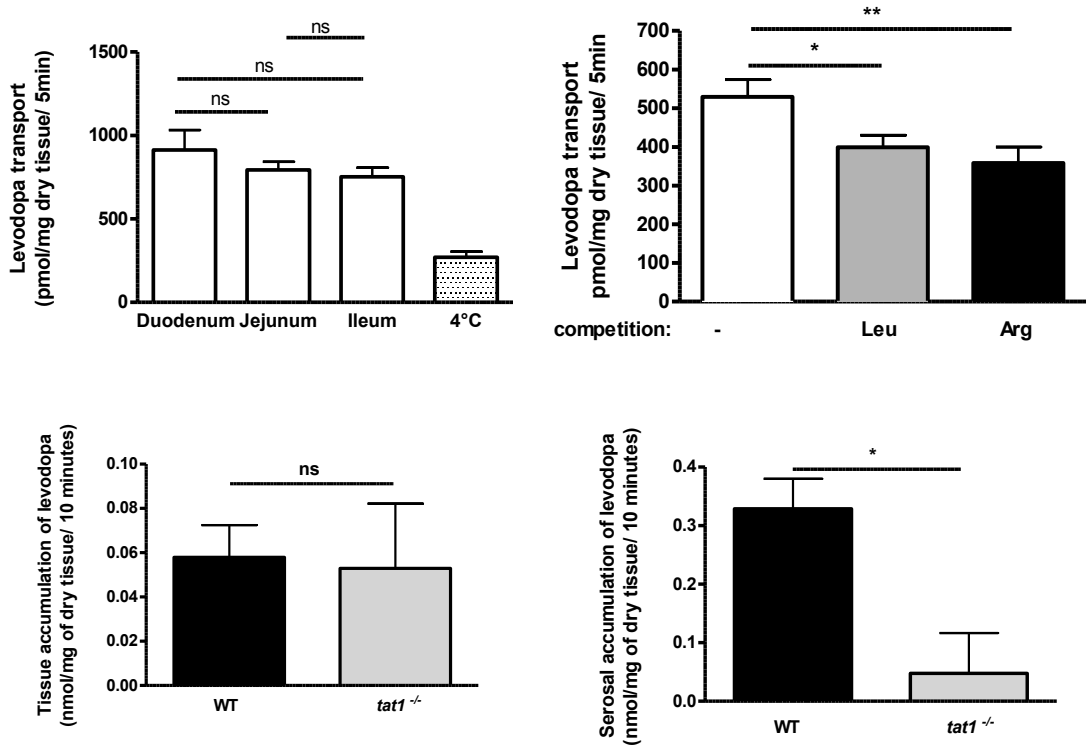
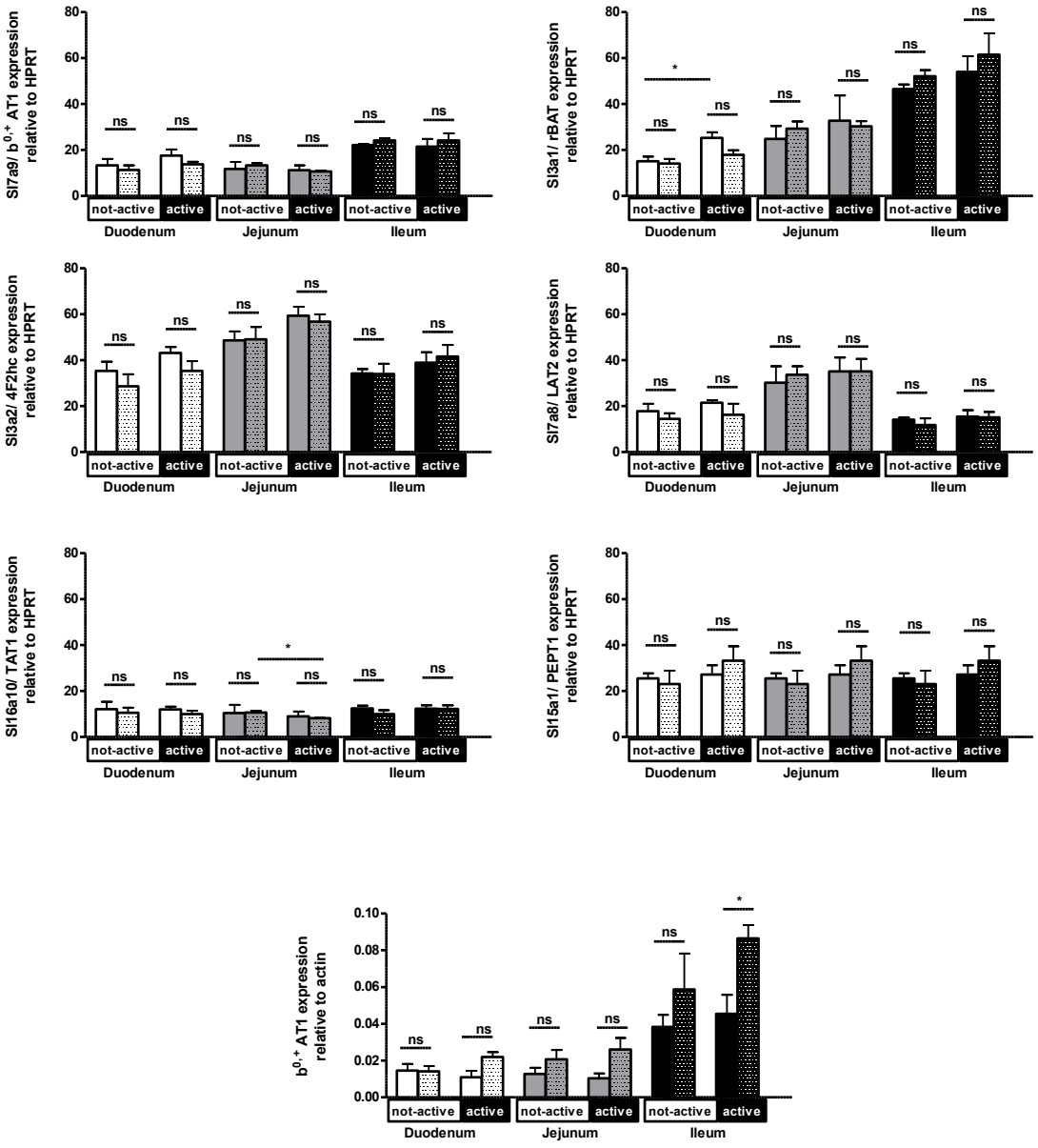

Levodopa transport
(pmol/mg dry tissue/ 5min)
1500
1000
500
0
ns
ns
ns
Duodenum
Jejunum
Ileum
4°C
Levodopa transport
pmol/mg dry tissue/ 5min
700
600
500
400
300
200
100
0
**
*
competition:
-
Leu
Arg
Tissue accumulation of levodopa
(nmol/mg of dry tissue/ 10 minutes)
0.10
0.08
0.06
0.04
0.02
0.00
ns
WT
tat1 -/-
Serosal accumulation of levodopa
(nmol/mg of dry tissue/ 10 minutes)
0.4
0.3
0.2
0.1
0.0
*
WT
tat1 -/-

**Figure 5**


Sl7a9/ b0,+ AT1 expression relative to HPRT
Sl3a1/ rBAT expression relative to HPRT
Sl3a2/ 4F2hc expression relative to HPRT
Sl7a8/ LAT2 expression relative to HPRT
Sl16a10/ TAT1 expression relative to HPRT
Sl15a1/ PEPT1 expression relative to HPRT
b0,+ AT1 expression relative to actin
not-active
active
Duodenum
Jejunum
Ileum
ns
*

# 8. Conclusion and outlook

## 8.1. Conclusion

SLC family transporters that function as symporters (cotransporters) or antiporters (exchangers) use electrochemical gradients of inorganic (or organic) solutes as driving force to actively transport specific substrates across cellular membranes. About 400 genes have been identified that are grouped according to sequence similarities (of 20% or more) into 52 SLC families[38,42]. In polarized cells (such as small intestinal enterocytes), solute carriers are targeted to the luminal or basolateral membranes, where they mediate substrate influx or efflux, respectively. Substrate specificities of SLC transporters may be wide (as for instance of $B^0AT1$[37], accepting the bulk of neutral amino acids) or narrow (as for instance of SIT1[48]) and several transporters show overlapping substrate specificities. For example, both luminal amino acid transporters $B^0AT1$ and SIT1 accept glycine and proline (with low affinity to $B^0AT1$) as substrates. Similarly, the monosaccharide transporter GLUT2 which can transiently be inserted in the luminal membrane accepts the SGLT1 substrates glucose and galactose as well as the GLUT5 substrate fructose[102]. Furthermore, some SLC transporters (such as for instance PEPT1, PAT1 or $b^{0,+}AT$-rBAT) also recognize therapeutic agents as substrates. Since membrane permeation across the gastrointestinal tract is one of the critical determinants for oral drug bioavailability, it is essential to precisely understand localization and function of these SLC transporters.

The quantity of absorbed nutrients may be modulated to meet body needs. This type of intestinal adaption occurs at the physiological, cellular and molecular level and reduces the risk of malnutrition in cases of e.g. short bowel syndrome[169]. Different growth factors, including Inslulin-like growth factors, Human growth hormone, Epidermal growth factor, etc. were shown to mediate this small intestinal adaption[170].

The regulation of expression and activity of intestinal SLC transporters is poorly understood[171]. Factors that may affect SLC transporter expression and function include presence or absence of accessory proteins, functional interactions of amino acid transporters, transporters' substrates, gastrointestinal diseases, medications et cetera; The presence of the luminal dipeptidase ACE2 for instance is necessary for adequate $B^0AT1$ expression in the brush border membrane of intestinal enterocytes as shown in *ACE2* knock-out mice exhibiting almost abolished luminal membrane $B^0AT1$ expression[5]. Another example of limiting factor is the $Na^+/H^+$ exchanger NHE3 (SLC9A3) that maintains intracellular pH, and hence the transmembrane $H^+$-electrochemical gradient that is necessary to drive the function of $H^+$-coupled (drug) transporters including PAT1 and PEPT1[43]. SLC transporters were shown to functionally interact for the transport of specific substrates using heterologous expression systems: for instance, recycling of aromatic amino acids (such as phenylalanine) via T-type

aromatic amino acid transporter TAT1 was shown to allow the efflux of other neutral amino acids that are not transporterd by TAT1 (such as glutamine) via the L-type amino acid exchanger LAT2-4F2hc[135]. It seems expectable that the transporters' substrates might regulate transporter expression, as shown for the substrate fructose regulating transcription of fructose transporter GLUT5 expression. This regulation appears to be specific, since only GLUT5, but not GLUT2 or SGLT1 expression is affected by dietary fructose[99]. Similarly, intestinal amino acid transporter expression is known to be induced by increased dietary protein levels[171]. Per contra, AA transporter expression may also increase in response to incomplete absorption of dietary proteins[171] such as for instance in short bowel syndrome.

At the molecular level, different major signaling pathways were shown to regulate gene expression within the intestinal epithelium including the Hedgehog family members Forkhead Box (FOX) factors and Hepatocyte nuclear factors 1α and 1β[172]. Regulatory mechanisms that may mediate these expression changes include regulatory microRNAs binding to mRNA transcripts and epigenetic regulations including imprinting, DNA methylation, regulation of histone modifications, and chromatin remodeling[172], but the involved sensing mechanisms are not known.

Different diseases and medications were shown to alter intestinal SLC transporter expression and function. Diseases affecting the intestinal microclimate pH such as Celiac disease or enterotoxin exposure reduce absorption of $H^+$-coupled transporter (i.e. PAT1 or PEPT1) substrates including the therapeutic agents salicylate and folate[43]. Furthermore, intestinal SLC mRNA levels were shown to be dysregulated in patients suffering from inflammatory bowel disease (IBD)[173]. Interestingly, PEPT1 expression was shown to be up-regulated in the large intestine of patients suffering from IBD, indicating, that the di- and tripeptide transporter plays a role in IBD pathogenesis. PEPT1 hereby transports colonic bacterial di- and tripeptides into colonocytes maintaining inflammation[174,175]. Among medications affecting intestinal SLC transporter expression and function, especially antidiabetics (including rosiglitazone, metformin and insulin) were shown to alter gene and protein expression of monosaccharide transporters and also of peptide transporter PEPT1[14,60,176].

During my PhD I focused on the regulation of intestinal SLC transporter expression in humans suffering from certain diseases (such as Parkinson's disease or type 2 diabetes mellitus) and/or in patients treated with certain medications. The transport of dietary contents across the small intestinal epithelium displays one of the first steps of interaction between ingested solutes (i.e. amino acids, monosaccharides, drugs, etc.) and the human body. Several diseases relate to diet (such as for instance type 2 diabetes mellitus) and also the absorption of several medications is influenced by dietary compositions. With this PhD work we hence intended to assess in humans the effect of medications such as ACE-inhibitors, angiotensin II $AT_1$ receptor blockers and metformin), of diseases such as type 2 diabetes

mellitus) as well as of dietary transporter substrates (i.e. dietary amino acids and dietary glucose overload in type 2 diabetes mellitus) on small intestinal amino acid and monosaccharide transporter expression and function.

In one observational study, the effect of chronic ACE-inhibitors and angiotensin II $AT_1$ receptor blockers intake on intestinal ACE2 and associated transporters expression was assessed. This study first confirmed that also in human ACE2 acts as an accessory protein for distinct amino acid transporters of the SLC6 family expressed in small intestine and interestingly showed that ACE2 gene and protein expression was modified in patients treated chronicaclly with these drugs. The underlying observation for another study was that dietary monosaccharides, experimental diabetes mellitus and metformin treatment had been shown to affect intestinal monosaccharide transporter expression and function in rodents[17,20,61-65]. We thus tested whether also in human subjects type 2 diabetes mellitus and/or metformin treatment also alter small intestinal monosaccharide transporter expression and hence probably also intestinal glucose absorption. Starting point for a further study was the fact that the main therapeutic agent for Parkinson's disease, levodopa, is a neutral amino acid. We studied its intestinal absorption mechanism and, since different amino acids may compete for its intestinal absorption, we tested the effect of routinely co-administered drugs (i.e. carbidopa or bensirazide) and of dietary amino acids on intestinal absorption.

We could hereby show that dietary compositions may affect the absorption of specific drugs (such as neutral amino acids affecting intestinal levodopa transport), that certain medications affect the expression pattern of intestinal SLC transporters (such as ACE inhibitors affecting the brush border membrane expression of $B^0AT1$, PAT and PEPT1), and that certain diseases (such as diabetes mellitus type 2) affect small intestinal hexose transporter GLUT2 expression.

## 8.2. Open questions

### Human intestine luminal ACE2 and amino acid transporters are regulated by ACE-inhibitors

***SIT interacts with ACE2 and collectrin.*** We could show a functional interaction of the system IMINO transporter SIT1 and the accessory proteins ACE2 and collectrin (TMEM27), similarly as previously shown for the named accessory proteins and neutral amino acid transporter $B^0AT1$[5,46]. ACE2 and collectrin hereby seem to increase SIT1-mediated L-proline transport via increasing the transporters' cellular surface expression without changing the apparent affinity to its transported substrate.

***Does ACE2-deficiency affect small intestinal SIT1 expression?*** It remains to be investigated, whether ACE2-deficiency decreases small intestinal SIT1 expression in vivo.

Unfortunately, a specific antibody against mouse SIT1 is still lacking[177] and therefore SIT1 expression in the *ACE2 knock-out* mouse could not be measured yet.

***Treatment with ACE-inhibitors increases small intestinal gene expression of ACE2, $B^0$AT1, PAT1 and PEPT1.*** Furthermore, our findings show an association of long-term ACE-inhibitor treatment with an increased gene expression of small intestinal ACE2 and of luminal amino acid and peptide transporters $B^0$AT1, PAT1 and PEPT1 and thus suggest a causal relationship. The question remains open whetherthe increased gene expression of ACE2, $B^0$AT1, PAT1 and PEPT1 upon ACE-inhibitor treatment is functionally relevant in terms of amino acid absorption and homeostasis. Indeed, blood plasma and urine amino acid levels as detected by high performance liquid chromatography (HPLC) analysis were not different between treated and non-treated patients. This is however not surprising, in view of the small number of subjects and the interindividual differences and because blood plasma and urine AA levels are subjected to many different other influencing factors, including diet as well as liver- and muscle metabolism[178]. Follow-up studies performing experiments such as stable isotope uptakes in human subjects or animals would have to be executed to answer this question.

***Does the increased small intestinal gene expression of transporters $B^0$AT1, PAT1 and PEPT1 have an effect on drug absorption?*** Since several drugs are transported via the amino acid and peptide transporters PAT1 (as e.g. vigabatrin or 5-aminolaevulinic acid) and PEPT1 (as e.g. β-lactams, angiotensin converting enzyme inhibitors or valacyclovir)[43], it would be interesting to perform a follow-up study assessing a potential impact of ACE-inhibitor treatment on intestinal drug absorption. It can be speculated that ACE-I treatment affects the pharmacokinetics of these drugs. Labeled drug uptake rates and/or post-intake plasma levels of non-labeled drugs or metabolites in patients under ACE-inhibitor treatment versus non-treated controls would have to be measured to test this hypothesis.

***Why does ACE-inhibitor treatment increase small intestinal ACE2, $B^0$AT1, PAT1 and PEPT1 mRNA expression?*** As discussed in the manuscript, the mechanism by which ACE-inhibitors might increase the expression of these genes is not clear, but an increase in ACE2 upon ACE-inhibitor treatment has previously been observed in different organs of rodents including heart, kidney and large vessels[6,7,179]. The involved regulatory pathway(s) are not known and the possible mechanisms mediating the mRNA upregulation include transcriptional activation, RNA stabilization and epigenetic regulations.

As the mRNA expression of ACE2 correlated with the gene expression levels of $B^0$AT1, PEPT1 and PAT1, a comparative promoter analysis was performed in order to identify potential common regulatory sequences. Hereby, common promoter modules with conserved transcription factor binding sites were found. Screening of the human promoter database for the occurrence of these modules revealed its presence only in 7 other genes, whereof only 4

genes showed small intestinal mRNA expression levels reliably quantifiable by qPCR. The product of none of these genes was increased in ACE-I-treated patients, indicating that these modules are per se not sufficient to mediate the ACE-induced regulation, but not excluding their involvement.

Hepatocyte nuclear factors 1a (HNF1a) and 4a (HNF4a) are transcriptional activators expressed in different organs including pancreas, liver, kidney, and intestine and are known to participate to the regulation of the expression of many tissue-specific genes,in particular for their differentiation-induced expression in enterocytes. Furthermore, both transcription factors were shown to induce $B^0AT1$ expression, whereas the ACE2 promoter could be activated only by HNF1a[98]. Hence, the expression of HNF1a and HNF4a was assessed in ACE-I treated patients and compared. The results of these experiments were pending, when the present thesis was written.

## Intestinal monosaccharide transporter expression in diabetic patients – effect of metformin treatment

***mRNA levels of GLUT2 were increased almost 2-fold in human small intestine in non-treated diabetics when compared to metformin-treated diabetics and non-diabetic controls, respectively. Why is the expression of GLUT2 increased diabetic patients?*** Of course, similar regulatory mechanisms as those involved in ACE2, $B^0AT1$, PAT1 and PEPT1 gene expression regulation upon ACE-I treatment (including transcriptional activation, RNA stabilization and epigenetic regulations) may cause GLUT2 up-regulation in diabetes mellitus type 2.

Since in kidney of diabetic rats, hepatocyte nuclear factors 1a, 3b and 4b (HNF-1a, HNF-3b and HNF-4a) were shown to play a substantial role in the overexpression of the GLUT2 gene[112,113], the expression of these transcription factors was assessed. Results of this experiment are pending when the present manuscript was written.

In experimental diabetes, but also in fructose- and/or glucose rich diets in rodents, the basolateral high-capacity, low-affinity facilitative glucose, fructose and galactose transporter GLUT2 also shows (transient) expression in the apical enterocyte membrane[18]. Whether the increased small intestinal GLUT2 mRNA expression observed in the present study is associated with a (transient) luminal transporter expression is being investigated by using immunofluorescence to visualize the subcellullar GLUT2 protein localization in the biopsy specimens. The results of these experiments were still lacking, when the present report was written.

***Metformin-treatment decreased small intestinal GLUT2 mRNA to normal expression levels (as measured in non-diabetic controls). Might this effect reflect a new mechanism of metformin action?*** In the present study only the gene expression of GLUT2

was measured. Furthermore, protein expression was visualized by immunofluorescence, which only allows a semi-quantitative analysis of protein expression (results pending when the present report was written). '*Less*' GLUT2 transporter mRNA as observed in diabetic patients under Metformin treatment does not necessarily mean '*less*' transport, especially not in a redundant system such as the small intestine. Hence, follow-up studies assessing the absorption of radiolabeled GLUT2-substrates glucose, galactose and/or fructose would be necessary to answer this question. Nevertheless, assuming that metformin-treatment affects small intestinal monosaccharide absorption as shown in rats, where metformin significantly decreased glucose absorption in the perfused intestine[14] we may only speculate whether metformin directly affects intestinal SLC transporter expression or whether it is the lower plasma glucose levels that influence intestinal monosaccharide transporter expression. Interestingly, metformin mostly accumulates in the small intestine after oral administration suggesting the digestive tract being a relevant site of metformin action[180].

GLUT5 gene expression showed a similar trend as GLUT2 with a tendency of increase in non-treated human type 2 diabetics (when compared to non-diabetics and to metformin-treated type 2 diabetics) without yielding statistical significance. ***Is GLUT5 expression altered in human type 2 diabetics or not?*** Unfortunately, the present study was a pilot study and thus a power analysis was not feasible. The group of non-treated type 2 diabetics consisted of only n= 5 individuals because most diabetic patients undergoing gastroduodenoscopy during the time of the study were under treatment. Hence the GLUT5 gene expression would have to be tested in a larger cohort of patients to obtain a clear conclusion.

## The molecular mechanism of intestinal levodopa absorption and its implications on treatment of Parkinson's disease

As expected, neutral amino acids competed for $b^{0,+}$AT-rBAT-mediated levodopa transport across the luminal enterocyte membrane. However, preloading cells with dibasic amino acids which are exchange substrates for $b^{0,+}$AT-rBAT mediated levodopa transport, did not significantly transstimulate the uptake of levodopa by this obligatory exchanger).

***May protein-sensitive fluctuating motor response to levodopa treatment in patients with end-stage parkinsonism be explained by competition of levodopa and dietary amino acids for small intestinal absorption?*** As shown in the present study ex-vivo using mouse everted rings as well as applying the heterologous expression system *X. laevis* oocytes, $b^{0,+}$AT-rBAT substrates (Leu, Arg) compete for $b^{0,+}$AT-rBAT-mediated levodopa transport. Accordingly, it was shown, that low-protein diets ameliorate daily motor fluctuations in end-stage Parkinson patients with severely depleted dopamine stores. Similarly, perfusion of human small intestine with L-leucine decreased intestinal levodopa absorption[69]. We

hypothesize that competition for $b^{0,+}$AT-rBAT mediated levodopa transport across the brush border membrane at least in part explains the reduction of fluctuating motor symptoms in end-stage Parkinson patients upon protein-restricted diets.

***May a transstimulation of small intestinal enterocytes with dibasic amino acids (as e.g. L-arginine) increase levodopa absorption?*** Since $b^{0,+}$AT-rBAT is an amino acid heteroexchanger[91], it seems to be evident, that preloading cells (small intestinal enterocytes) with dibasic $b^{0,+}$AT-rBAT-substrates (such as L-arginine) would increase neutral amino acid (such as levodopa or L-leucine) uptake by so-called transstimulation. As suggested by Pineda and co-workers in 2004 using the two electrode voltage clamp technique in *X. laevis* oocytes[181], preloading oocytes with Arg indeed increased Leu transport and vice versa. In contrast, when we preloaded oocytes with L-arginine (as shown in the present study), there was only a non-significant increase in L-leucine or levodopa uptake measured by radiolabeled intracellular substrate accumulation. Whether these diverse findings reflect technical limitations, may not be answered, since Pineda et al. did not measure radiolabeled substrate uptake and per contra we did not utilize the by two-electrode voltage clamp technique. Of course, *X. laevis* oocytes endogenously contain dibasic amino acids[78,182], and it is not clear to what extent this endogenous level suffices to fully trans-stimulate the uptake of extracellular substrates. Transferred to Parkinson patients – assuming that Pineda and co-workers were right – one might expect that protein-rich meals 1 or 2 hours before levodopa intake could increase levodopa uptake by transstimulation. But, (as suggested by Pineda et al.[181]) whereas transstimulation with the dibasic amino acid Arg increased Leu uptake, L-leucine preload appeared to decrease transcellular Leu transport, observations that are supported by our data obtained with ex-vivo mouse everted rings as well as using *X. laevis* oocytes. Hence, a L-arginine-rich- and L-leucine-poor diet would be necessary to transstimulate levodopa absorption in vivo, assuming intracellular dibasic amino acid levels in small intestinal enterocytes are below the saturation level of $b^{0,+}$AT-rBAT.

## 8.3. Outlook and personal statement

During my PhD I intended to test the impact of dietary nutrients (i.e. amino acids), of drugs (i.e. ACE-inhibitors, angiotensin II $AT_1$ receptor blockers, metformin) and of disease (i.e. type 2 diabetes mellitus) on small intestinal nutrient (i.e. amino acid and monosaccharide) transporter expression. To assess possible regulatory mechanisms underlying the discovered changes in expression patterns, expression of transcription factors were assessed (ongoing) and/or ex-vivo experiments were performed.

We hereby found that chronic intake of ACE-inhibitors increases small intestinal expression of distinct amino acid transporters and of intestinal ACE2. Furthermore, a functional interaction

of IMINO transporter SIT1 and of accessory protein ACE2 was shown for the first time. In addition, type 2 diabetic patients were shown to exhibit increased intestinal GLUT2 transporter mRNA expression, an effect that was annihilated by metformin treatment. Finally, we could identify the small intestinal amino acid transporters responsible for absorption of the main Parkinson drug levodopa and dietary neutral amino acids were shown to compete small intestinal levodopa absorption.

Due to my personal intention to pursue an academic career in pediatric surgery I aim to continue my research career undertaking translational research. Hence, the subsequent two follow-up projects were started adjacent to my PhD thesis:

The first project intends to assess the ***impact of small intestinal atresia on amino acid and monosaccharide transporter expression in the human newborn gut:*** An interesting model to assess the effect of dietary nutrients on intestinal nutrient transporter expression constitutes the human newborn suffering from small intestinal atresia. It is hypothesised that small intestinal atresia (referring to a complete obstruction of the small intestine) occurs secondary to an ischemic insult in the later pregnancy or primary to a malformation in the early pregnancy[183]. Intestinal segments distal to congenital small intestinal obstruction have been suggested to be immature due to lack of luminal access of amniotic fluid, that contains inter alia carbohydrates, amino acids, peptides, proteins, lipids and multiple growth factors[184,185]. In collaboration with the Children's Hospital of Zurich, we started to sample tissue samples proximal and distal to the atretic segment. The preliminary analysis of genes and proteins assessed in 4 human newborns (aged 1 to 4 days) suffering from small intestinal atresia indicates a reduced gene expression of some basolateral amino acid- as well as of luminal hexose transporters GLUT5 and SGLT1 in the small intestine distal to the atretic segment.

The second project examines the ***amino acid and monosaccharide transporter expression in colorectal cancer and kidney cell carcinoma:*** Tumor cells require a high and constant supply of nutrients to support their characteristic unabated growth. Amino acids provide the primary source of cellular nitrogen, which is used for nucleotide-, glutathione-, and protein synthesis. Monosaccharides are used as an oxidative fuel source for ATP generation. To fulfil these metabolic needs, tumor cells must express large amounts of specific nutrient transporters on their cellular membranes[186].
AA transporter LAT1 (L-type amino acid transporter 1; SLC7A5) is responsible for large neutral amino acid transport and has been shown to be highly expressed in different tumors, including prostatic-, oesophageal-, gastric-, pancreatic- and pulmonary carcinoma [186,187]. It has thereof been indicated, that LAT1 serves as prognostic predictor, diagnostic tool and specific molecular target in cancer diagnostics and therapy. In addition, CD98hc (SLC3A2), a type II transmembrane cell surface molecule, has been described in different tumors, including melanoma, squamous cell carcinoma of the larynx and adenocarcinomas of the

lung [188].

The diagnostic of colorectal cancer mainly consists of ileo-colonoscopy, whereas kindey cell carcinomas are often an incidental finding in abdominal CT scans executed for different reasons. The main therapeutic option in both, colorectal cancer and kindey cell carcinoma, still consists of complete surgical removal, which may be followed by other therapies, including chemotherapy and radiation[189].

Whereas some amino acid transporters/transporter subunits have been shown to be highly expressed in different tumor cells (see above), the “transportome” of colorectal cancer- and kidney cell carcinoma cells has not been identified yet. Since amino acid and monosaccharide absorption in small intestine and re-absorption in kidney proximal tubule is provided by different transport proteins, that must functionally interact in order to guaranty net unidirectional amino acid and monosaccharide transport[1,135], we assume several amino acid and monosaccharide transporters being highly expressed in colorectal cancer and kidney cell carcinoma cells.

In collaboration with the division of Pathology of the University Hospital of Zurich, we hence aim to assess the tumor transportome in human colorectal cancer and human kidney cell carcinoma.

# 9. Curriculum vitae

## Personal Data


| | |
|---|---|
| Name | VUILLE-DIT-BILLE Raphael Nicolas |
| Date of birth | 13th of December 1980 |
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## Education

| | |
|---|---|
| 11/2012 | 'Basisexamen Chirurgie' (basic exam in Surgery for board certification) |
| 2008 – 2010 | PhD-student at the Institute of Physiology, University of Zurich, Switzerland, in the group of Prof. F. Verrey |
| 2005 – 2011 | Medical thesis (MD) under the supervision of Prof. J. Stover |
| 2008 – 2010 | USMLE (United States Medical Licence Examination) Steps 1, 2 CS (clinical skills) and 2 CK (clinical knowledge) |
| 2005 – 2007 | Studies of Human Biology at the University of Zurich (as a part of the MD-PhD program under the supervision of Prof. A. Aguzzi) |
| 2000 – 2007 | Studies of Medicine at the University of Zurich |
| 1993 – 1999 | 'Gymnasium, Typus A' at the cantonal school of Zurich Wiedikon |

## Work

| | |
|---|---|
| 04/2013 – now | Resident in Pediatric Surgery at the University Children's Hospital of Zurich (Prof. M. Meuli) |
| 01/2011 – 02/2013 | Resident in General Surgery at the Hospital of Bülach, Zurich (Dr. B. Muff) |

## Awards/Specials

| | |
|---|---|
| 2008 | Research Grant by the Swiss National Fonds (180'000.- CHF for three years) |
| 2000 | Admission to the Swiss Study Foundation (Furtherance of gifted students); Alumnus since 2010 |

| | |
|---|---|
| 2000 | Numerus Clausus (entry exam Studies of Medicine): 98.9% percentile |
| 1999 | Best male finisher of the high school |

## Publication list

**Vuille-dit-Bille** RN, Sauter D, Pfofe D, Zagralioglu O, Jandali AR, Nadig J, Dinçler S, Muff B. High-grade cutaneous angiosarcoma of the breast 8.5 years after radiotherapy. Breast J. 2013 Jul-Aug;19(4):435-6. doi: 10.1111/tbj.12130. Epub 2013 May 31. **Case report.**

**Vuille-Dit-Bille** RN, Ha-Huy R, Stover JF. Changes in plasma phenylalanine, isoleucine, leucine, and valine are associated with significant changes in intracranial pressure and jugular venous oxygen saturation in patients with severe traumatic brain injury. Amino Acids. 2012 Sep;43(3):1287-96. doi: 10.1007/s00726-011-1202-x. Epub 2011 Dec 22. **Original scientific article.**

**Vuille-Dit-Bille** RN, Ha-Huy R, Tanner M, Stover JF. Changes in calculated arterio-jugular venous glutamate difference and SjvO2 in patients with severe traumatic brain injury. Minerva Anestesiol. 2011 Sep;77(9):870-6. **Original scientific article.**

Verrey F, Singer D, Ramadan T, **Vuille-dit-Bille** RN, Mariotta L, Camargo SM. Kidney amino acid transport. Pflugers Arch. 2009 May;458(1):53-60. doi: 10.1007/s00424-009-0638-2. Epub 2009 Jan 28. **Review.**

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